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Cholinergic Interneurons and Synaptic Reorganization within the Nucleus Accumbens Shell and Core: Potential Neural Substrates Underlying Drug Addiction

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by

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Dedication

This is for you, Mom and Dad.

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Cholinergic Interneurons and Synaptic Reorganization within the Nucleus Accumbens Shell and Core: Potential Neural Substrates Underlying Drug Addiction

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Drug abuse and dependence are among the most challenging public health issues facing America today. The acute treatment of drugs of abuse such as psychostimulants (Trantham-Davidson and Lavin, 2004) and opiates (Harris and Williams, 1991) produce transient changes in cellular activity and synaptic signaling. Repeated drug treatment, however, results in persistent cellular and behavioral changes, such as altered dendritic morphology and behavioral sensitization (Robinson and Kolb, 1999b). Synaptic changes in the brain are posited to underlie a repertoire of drug-induced persistent behaviors, including sensitization, psychosis and relapse. Direct evidence of drug-induced synaptic plasticity, however, has not been demonstrated. The present studies were designed to examine cholinergic neurons and synaptic rewiring as potential neural substrates involved in acute and chronic drug exposure. The proposed studies tested the hypotheses that 1) cholinergic interneurons within the nucleus accumbens (NAcc) are activated by the acute

self-administration of cocaine, 2) dopamine (DA) D5 and D2 receptors localized on cholinergic interneurons potentially undergo cocaine-induced neuroadaptation, and 3) repeated administration of cocaine leads to an increase, while repeated administration of morphine leads to a decrease, in the number of synapses within the NAcc, whereas an increase in the number of synapses occurs in the NAcc core of animals exhibiting behavioral sensitization.

These studies revealed that accumbal cholinergic interneurons are activated by acute cocaine self-administration and elucidate the specific localization of DA receptor subtypes, D5 and D2, on these cells, suggesting their potential role in mediating drug-induced DA changes within the NAcc. The final study provided the first ultrastructural evidence that an increase in the number of excitatory synapses in the NAcc shell occurs following 4-weeks of cocaine and morphine treatment followed by 3 weeks abstinence and that cocaine sensitization is associated with an increase in the number of excitatory synapses in the NAcc core. These findings provide the groundwork for future studies examining the precise cellular and synaptic substrates underlying a repertoire of drug-induced behaviors that contribute to the persistence of addiction. Improved pharmacotherapeutic and behavioral treatments can then target the specific cellular and synaptic microcircuitry critically involved in the different stages of drug abuse and dependence.

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Chapter 1: Introduction

Drug abuse and dependence are two of the most challenging public health issues facing America today. The Office of National Drug Control Policy estimates illicit drug trafficking and abuse costs the United States 180.9 billion dollars each year (National Drug Intelligence Center, 2006). Furthermore, drug abuse takes an enormous emotional toll on the families struggling to help the people they love. Research studies aimed at identifying possible neuronal substrates and mechanisms underlying drug abuse and dependency are helping to combat this national health problem. The better we understand the cellular circuits and neuroadaptive changes underlying compulsive drug use, the better chance we will have at treating drug dependency successfully. The following research studies attempt to broaden the current knowledge surrounding the effects of acute and chronic drug treatment by shedding light on potential neuronal substrates mediating the actions of addictive drugs in the brain.

Drugs of abuse activate pleasure centers in the brain and increase levels of dopamine (DA), the neurotransmitter responsible for the euphoric effects or "high" described by many cocaine users (Volkow et al., 1997). Acute drug administration leads to increased DA release and neuronal activation in the NAcc (Moratalla et al., 1996; Gerrits et al., 2002). Recently, however, DA's role in drug abuse and dependency has become increasingly unclear, as more studies are beginning to show that animals continue to show preference for drugs after DA signaling has been pharmacologically blocked, destroyed, or genetically inactivated (Berridge and Robinson, 1998; Robinson et al., 2005). The effects of drug administration on glutamatergic signaling in key drug-related brain regions are receiving more attention as a result of the significant role the PFC plays in self-administration and drug-seeking behavioral paradigms (Sun and Rebec,

2006). Thus, the search for one neurotransmitter acting at the center of drug addiction may be too simplistic an approach. Therefore it is likely that the contribution of both neurotransmitter systems (i.e. DA and glutamate) mediate the actions of drugs of abuse, utilizing the integrative properties of the cells located in these critical drug-associated brain regions.

Repeated drug use can lead to long-lasting molecular and cellular changes in the brain detectable even after drug cessation (Carlezon et al., 1998; Robinson and Kolb, 1999b; Vanderschuren and Kalivas, 2000; Shaw-Lutchman et al., 2002; Norrholm et al., 2003; Chao and Nestler, 2004). These neuroadaptive changes presumably underlie the persistent behavioral changes observed following chronic drug treatment (Robinson and Kolb, 1999a, b; Bibb et al., 2001; Wang et al., 2004; Chen and Chen, 2005). Although, when these molecular changes are mapped along the same time course as behavioral changes, studies show that the behaviors outlast the molecular changes. For example, one of the longest lasting changes in the brain detected following repeated drug treatment is the accumulation of delta FosB, but once drug treatment ceases, delta FosB protein levels return to baseline after 6-8 weeks. Drug-related behavioral changes, such as sensitization, can persist for years (Paulson et al., 1991), long after delta FosB levels have subsided. Therefore, a change in synapse number, a long-lasting neuroadaptive change in the brain which has been previously found to occur following learning paradigms, may help explain the cellular mechanisms underlying persistent drug-induced behaviors.

The following studies were designed to address questions that remain unanswered in the literature regarding acute and chronic effects of drug administration on neuroplasticity. Critical brain areas and molecular changes involved in addiction have been previously identified, but specific cell types within these brain regions and their role in addiction have not been previously examined. Therefore, the first two studies

comprised in this dissertation examine a specific cell population in the NAcc, the cholinergic interneurons, a group of cells in the NAcc that have remained largely overlooked in drug addiction research. Furthermore, these studies provide the first evidence of synaptic changes in the NAcc associated with repeated drug treatment. While key brain areas and molecular changes following long-term drug administration have been identified, cellular neuroadaptations underlying drug-induced persistent behaviors remain unclear. It has been posited that such long-lasting behaviors may be explained by synaptic reorganization. Thus, synaptic changes resulting from long-term drug administration, as reported in the final chapter of this dissertation, may be one type of neural substrate mediating long-lasting drug-related persistent behaviors. The following studies were designed to test the hypotheses that 1) cholinergic interneurons in the NAcc shell and core are differentially activated by the acute self-administration of cocaine, 2) DA D5 and DA D2 receptors are localized on cholinergic interneurons of the NAcc shell and core and these DA receptors potentially undergo cocaine-induced neuroadaptation, 3) repeated administration of cocaine leads to an increase in the number of synaptic connections within the NAcc shell and core, while repeated administration of morphine leads to a decrease in the number of synaptic connections within the NAcc shell and core and 4) the increase in the number of synapses in the NAcc core are associated with animals exhibiting behavioral sensitization.

The remaining pages of this chapter provide the groundwork for the hypotheses presented in the studies discussed in chapters 2 through 5. The first sections of this introduction present common drug-related neurocircuits that are known to undergo neuroadaptive changes following the administration of most drugs of abuse, including psychostimulants and opiates, and the neuronal populations that make up these neurocircuits. Because the primary neuronal pathways activated by drugs of abuse

involve dopaminergic systems, the next sections discuss DA receptor families and subtypes and the intracellular cascades they activate, including activation of immediate early genes (IEGs), as a result of DA binding. These receptors, although not solely responsible for the long-lasting changes resulting from cocaine and morphine treatment, undoubtedly play a role in drug abuse and dependence. Lastly, the final sections present a review of the literature on the molecular actions of cocaine and morphine and the effects of long-term use. Specifically, these sections address the potential consequences of drug use and the structural neuroadaptations within the NAcc potentially underlying persistent drug-induced behaviors.

1.1 Mesolimbic Dopamine Pathway

The mesolimbic DA pathway consists of projections from DA neurons of the ventral tegmental area (VTA) to the NAcc (Fig. 1.1) (Bozarth, 1991). This neuronal circuit is the primary pathway underlying reward and reinforcement (Di Chiara and Imperato, 1988). The VTA also sends DA projections to the cortex, called the mesocortical DA pathway (Bozarth, 1991). While drugs of abuse have the ability to modulate many different neurotransmitter systems in the brain, increases in DA neurotransmission in the NAcc following the initial stages of drug intake is regularly observed. In fact, if the DA-containing cell bodies in the VTA are destroyed with 6-OHDA, animals significantly reduce their drug-intake or completely stop responding for drug administration (Roberts and Koob, 1982). Interestingly, if the postsynaptic actions of DA are blocked in the NAcc, via DA receptor antagonists, animals trained to self-administer for drugs will increase their drug intake, presumably, to compensate for the disregulation of DA (Stellar and Corbett, 1989). Recently, however, the idea of a central role for DA in drug abuse and dependence is being challenged. Evidence continues to

accumulate which suggests a significant role for glutamate (in addition to DA) in drug-related processes (Vanderschuren and Kalivas, 2000).

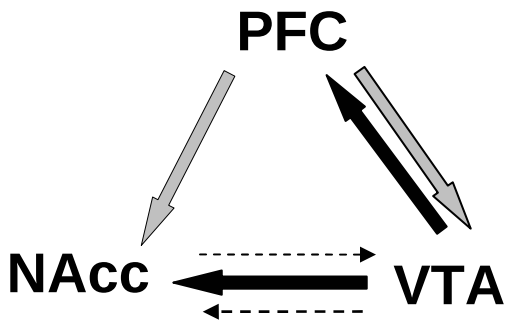

Figure 1.1. Mesolimbic and mesocortical projections. Simplified diagram showing dopaminergic (black arrows) and glutamatergic (gray arrows) projections within the mesolimbic (VTA-NAcc) and mesocortical (VTA-PFC) DA pathways, as well as GABAergic projections (dashed arrow).

1.2 Nucleus Accumbens

The NAcc is also known as the ventral striatum. This region of the striatum has been shown to possess many of the same cell types found in the dorsal striatum, namely the medium spiny projection neurons (MSNs), large aspiny cholinergic interneurons, as well as several types of inhibitory GABAergic interneurons (Kawaguchi, 1997). The NAcc as a whole is a neuroanatomically complex structure, integrating both limbic and motor information from mesencephalic DA sources and interconnected with other brain areas. More importantly, neuroanatomical and neurochemical studies have elucidated the heterogeneous nature of the NAcc shedding light on the range of functions of this particular brain region. The distinctions between the neuroanatomical, neurochemical and neuronal functions are outlined below.

1.2.1 Shell and Core Subcompartments of the Nucleus Accumbens

The NAcc is divided into two subcompartments, the shell and core. The shell compartment of the NAcc receives dense dopaminergic innervation from A10 DA neurons of the VTA (Voorn et al., 1986; Brog et al., 1993), while the core compartment of the NAcc receives its major source of DA from A9 DA neurons of the substantia nigra pars compacta (SNc) (Nirenberg et al., 1996). Such differences in dopaminergic innervation, as well as their afferent and efferent connections with other brain regions, account for the functional differences between these two subcompartments (Zahm and Heimer, 1990; Heimer et al., 1991; Di Chiara, 2002). Heimer et al. (1991) masterfully explored the neuroanatomical connections of the shell and core highlighting the efferent connections of the shell with the extended amygdala (limbic) and the core with the entopeduncular nucleus (motor). Furthermore, glutamatergic projections from the infralimbic PFC innervate the NAcc shell, whereas projections from the prelimbic PFC innervate the NAcc core (Ding et al., 2001). Moreover, the shell and core subregions can also be distinguished using specific neurochemical markers. For example, immunoreactivity for calbindin, used to distinguish between the patch (limbic) and matrix (motor) compartments, further supports the notion that the shell is more involved in limbic-related functions (Gerfen, 1984) as opposed to the core, which has been strongly linked to motor-related functions. However, upon closer examination, each subregion shows inhomogeneous staining along the rostral-caudal as well as the medial-lateral axes (Voorn et al., 1986; Meredith et al., 1992; Meredith, 1999), thereby adding to the neuroanatomical complexity within each subcompartment of the NAcc.

Drugs of abuse differentially activate these two divisions of the NAcc. Studies suggest that the NAcc shell plays an important role in incentive arousal because excitotoxic lesions to this brain area interfere with conditioned reinforcement (Parkinson

et al., 1999). Alternatively, excitotoxic lesions to the NAcc core disrupt instrumental performance (Corbit et al., 2001). Dopamine release in the NAcc shell is greater than in the core, with acute (Pontieri et al., 1995) administration of cocaine (independent of whether the drugs are self-administered or experimenter-administered). Caffeine, a non-addictive drug, does not produce the same DA response in the NAcc, thus, DA release in the shell is specific to drug-rewards (Acquas et al., 2002). Furthermore, chronically (15 days) self-administered cocaine shows an increase in DA in both the NAcc shell and core, with significantly higher levels of DA in the shell (Di Chiara et al., 2004). Additionally, pharmacological studies suggest that drug-induced changes in glutamate release in the NAcc and drug-mediated changes in behavior depend on dopaminergic systems (Reid and Berger, 1996; Reid et al., 1997). More recently, at the behavioral level, changes in the morphology of cells in the NAcc core have been correlated with behavioral sensitization (Li et al., 2004).

1.2.2 Medium Spiny Projection Neurons of the Nucleus Accumbens

Medium spiny projection neurons (MSNs) are the major output neurons of the NAcc, making up 95% of the total striatal cell population. These GABAergic neurons project to the ventral pallidum, entopeduncular nucleus and the mesencephalon (Heimer et al., 1991). They typically alternate between two electrical states, the Up state (i.e. depolarized state) and the Down state (i.e. polarized state), where action potentials only arise during the Up state (Bennett et al., 2000). Their cell bodies range in size from 15-20 microns and these cells have dendrites that can extend up to 500 microns. Dendrites of MSNs are spine-free for the first 20 microns and then are covered in spines up to the terminal tips of the dendrites (Wilson and Groves, 1980). The highest density of spines occurs at approximately halfway along the length of the dendrite, about 40 to 60 microns away from the cell body (Meredith et al., 1992). These cells receive glutamatergic

(excitatory) inputs from the prefrontal cortex, thalamus, hippocampus, and the amygdala primarily onto the heads of their dendritic spines. Dopaminergic afferents also synapse onto the spines of these neurons, although these synapses are typically observed on the necks of the spines, dendritic shafts and sometimes the cell body (Zahm, 1992). Differences in the morphology of MSNs exist across the two compartments of the NAcc shell and core. For example, MSNs in the shell are smaller than cells in the core and have fewer dendritic branches as well as a lower spine density (Meredith et al., 1992).

1.2.3 Aspiny Cholinergic Interneurons of the Nucleus Accumbens

The cholinergic interneurons of the NAcc are some of the largest cells in this brain region. These interneurons can be identified using antibodies directed against choline acetyltransferase (ChAT), an enzyme used to synthesize acetylcholine (ACh). Cholinergic interneurons make up 1-2% of the total striatal cell population, and have large somata (20-50 μm) and extensive dendritic trees that extend up to 1 mm in length. These interneurons are also characterized by bifurcating varicose axons that form a dense axonal plexus (Wilson et al., 1990). The large dendritic and axonal fields allow these cells to integrate a variety of inputs over long distances within the NAcc. Although these cells typically do not have spines on their dendrites, spine-like appendages are sometimes observed on distal portions of their dendrites (Wilson et al., 1990). Furthermore, electrophysiological studies have shown that cholinergic interneurons have resting potentials that are close to threshold (i.e. readily excitable), and these cells typically fire in an irregular tonic pattern (Wilson et al., 1990). These cells are also shown to receive direct synaptic contact from the thalamus (Lapper and Bolam, 1992). Other glutamatergic brain areas that innervate the NAcc include the PFC, amygdala, and the hippocampus (Kalivas, 2004), which may also form synapses onto these cells. Thus, it has been

suggested that these cells may specialize in mediating information from the thalamus to the NAcc.

1.3 Dopamine Receptors

The DA receptor family is a group of five unique metabotropic, or G-protein-coupled, receptors (D1, D2, D3, D4, and D5) with seven transmembrane regions. These receptors are divided into two distinct subfamilies, the DA D1-like receptors (D1 and D5) and the DA D2-like receptors (D2, D3 and D4). Dopamine D1 receptors are characterized by their ability to activate adenylyl cyclase (AC), while DA D2 receptors inhibit the stimulation of AC (Missale et al., 1998). Such functional differences are typically attributed to differences in structure between the two receptor families. One of the more prominent differences between the receptor subfamilies is the length of their third intracellular loop and carboxyl terminal tail. For example, D1-like receptors have a short third intracellular loop and a long carboxyl terminal tail, while D2-like receptors have long third intracellular loops and a short carboxyl terminal tail. The third intracellular loop of DA receptors are known to couple to specific types of G proteins (inhibitory versus stimulatory), thereby accounting for some of their functional differences (Ilani et al., 2002). When DA binds to one of these receptors, the receptor undergoes a conformational change. This conformational change leads to activation of the G-protein and subsequent second messenger proteins. One other distinguishing property between the two receptor families is the presence of introns in the receptor genes (Civelli et al., 1993). Dopamine D1 receptor genes (both D1 and D5 receptors) lack introns. The DA D2 receptor family contains introns in its receptor genes, and this is believed to be responsible for the generation of receptor variants, such as the long and short chain variants of the DA D2 receptor subtype (O'Dowd, 1993). Interestingly, although these receptor subfamilies exhibit unique structural differences and opposing functions, upon

activation via DA binding, these receptors will in some cases produce a synergistic effect. Thus, DA receptors are important neuronal substrates, and prove to be critical targets when treating a variety of neurological disorders including schizophrenia, Parkinson's disease and drug dependency.

1.3.1 Dopamine D1 Receptor Subfamily

The DA D1 receptor family consists of DA D1 and DA D5 receptor subtypes. These receptors have highly conserved amino acid sequences, sharing 80% homology in their transmembrane domains. While these two receptor subtypes have similar pharmacological properties, they exhibit different binding affinities for the neurotransmitter DA. The DA D5 receptor has 10 times greater binding affinity for DA than the DA D1 receptor (Grandy et al., 1991). To date, there is no selective agonist or antagonist that distinguishes between the D1 and D5 receptor subtypes. Activation of either the DA D1 or the DA D5 receptor leads to increased levels of AC, via activation of the G-protein. In fact, cells selectively expressing DA D5 receptors exhibit higher levels of AC basal activity than cells expressing DA D1 receptors (Tiberi and Caron, 1994). Adenylyl cyclase then activates cAMP, which can activate targets such as, kinases and phosphatases. These proteins, in turn, can activate (or inactivate) calcium and potassium ion channels or other cell surface receptors, thereby affecting the membrane potential of the cell and subsequent neurotransmitter release, such as ACh from cholinergic interneurons that highly express the DA D5 receptor. Repeated activation of these intracellular cascades can potentially lead to changes in neuroplasticity, such as long-term potentiation.

1.3.2 Dopamine D2 Receptor Subfamily

Dopamine receptors classified in the D2 subfamily include the DA D2, D3 and D4 receptor subtypes. Unlike the DA D1 receptor family, activation of receptors in the DA D2 receptor subfamily leads to a decrease in AC and cAMP levels. Dopamine binds to the DA D3 receptor subtype with an affinity that is 20 times greater than its binding affinity to the DA D2 receptor subtype (Sokoloff et al., 1990), presumably due to the structural differences in their third intracellular loops (Robinson et al., 1994). The DA D4 receptor, on the other hand, is most readily distinguished from the other D2 receptor subtypes by its high affinity for clozapine, a DA agonist (Van Tol et al., 1991). Dopamine D2 receptors, in particular, have been shown to act primarily as autoreceptors, localized on the presynaptic terminal, where they mediate DA release from dopaminergic nerve terminals. The cellular mechanism underlying DA D2 autoreceptor function is the ability of this receptor to induce the efflux of potassium from the cell. This outward potassium current leads to the hyperpolarization of the cell membrane, thereby inhibiting the release of DA from the axon terminal (Greif et al., 1995). A similar inhibitory mechanism on cell firing is postulated for cholinergic interneurons as well (Sil'kis, 2004). Furthermore, the DA D2 receptor subfamily has also been observed to mediate calcium currents, although the intracellular mechanisms are not completely understood.

1.3.3 Dopamine Receptor Distribution in the Nucleus Accumbens

Dopamine D1 and D2 receptors are the most predominant receptor subtypes in the striatum, including the NAcc (Gingrich and Caron, 1993). The MSNs in the NAcc express high levels of DA D1 and D2 receptors, although not likely expressed in equal proportion. In fact, there has been a long standing controversy over the expression of these two particular receptor subtypes on MSNs. These projection neurons have been divided into two distinct populations. One group of MSNs expresses high levels of DA

D1 receptors as well as substance P and projects primarily to the SN and internal segment of the globus pallidus (GPi). The second population of MSNs expresses high levels of DA D2 receptors and enkephalin and projects to the external segment of the globus pallidus (GPe) (Gerfen, 1992). Gerfen and colleagues continue to publish studies supporting the existence of these two pathways, which he refers to as the direct (D1-mediated) and indirect (D2-mediated) striatal projection pathways (Gerfen, 2000, 2003, 2006). Still, other studies report that MSNs projecting to the SN and GPi also send projections to the GPe, which suggests that these pathways are not discrete (Kawaguchi et al., 1990). Another line of evidence arguing against the separate MSN projection pathways is the subpopulation of MSNs (20-25%) that co-express D1 receptors, substance P, D2 receptors and enkephalin (Surmeier et al., 1996).

The remaining DA receptor subtypes, are expressed at lower levels in the NAcc, with the exception of the DA D3 receptor, which is found at higher levels in the NAcc than in the dorsal striatum (Schwartz et al., 1998). The expression of the DA D3 receptor appears to be more highly concentrated in the NAcc, olfactory tubercle and the minor islands of Calleja (Bouthenet et al., 1991). The DA D5 receptor, which is reportedly expressed at low levels in the NAcc, is found at high concentrations on cholinergic interneurons (Bergson et al., 1995; Yan and Surmeier, 1997), with little to no expression of the D1 receptor subtype (Le Moine et al., 1990). Finally, DA D4 receptors account for little of the DA D2-like binding in the NAcc (15%) or dorsal striatum (10%), as compared to other brain areas, such as the hippocampus (55%), dorsolateral (51%) medial prefrontal (47%) and entorhinal (42%) cortices (Tarazi et al., 1997).

1.4 Immediate Early Gene Expression

The IEG, *c-fos*, is activated quickly (~ 5minutes) following a stimulus, with protein levels peaking at about 2-3 hours and returning to baseline after 8 hours (Hope,

1998). The phosphoprotein product of the IEG *c-fos*, Fos, is located in the nucleus and can bind to other IEG proteins, such as Jun, forming the transcription factor AP-1 complex (Hope, 1998). The AP-1 complex then regulates the transcription of genes downstream. These proteins are known to be involved in the early portion of the signaling cascades needed for cell growth and differentiation. The induction of Fos expression during the early stages of learning has been previously reported to correlate with sites of synaptic plasticity after repeated treatment (Kleim et al., 1996). Moreover, a separate class of Fos-like proteins called chronic Fos-related antigens (FRAs) is induced following chronic, but not acute, drug administration (Hope et al., 1994; Nye et al., 1995; Nye and Nestler, 1996). The chronic FRAs are more stable than the Fos-like proteins induced acutely, which are less stable and short-lived. Delta FosB is a splice variant of the IEG FosB and accumulates after chronic drug treatment in a region-specific manner (Nestler et al., 1999). Delta FosB binds with Jun proteins to form stable and long-lasting AP-1 complexes, which are believed to mediate some of the long-term effects of drug abuse and dependency (Nestler et al., 2001).

1.5 Cocaine

Cocaine, a psychostimulant, comes from the leaves of the coca plant. In 1855, Friedrich Gaedcke, a German chemist, was the first person to isolate the cocaine alkaloid, which he named erythroxyline and published in the journal *Archives de Pharmacie*. Four years later, Albert Niemann, a graduate student at the time, studied the coca leaves at the University of Gottingen in Germany, where he worked to improve the purification process of erythroxyline, giving it the name cocaine. He published his findings on the purification process of cocaine in his dissertation in 1860 (Wikpedia, 2006). In 1884, Sigmund Freud published *On Coca*, advocating the medicinal properties of cocaine and claiming that cocaine can be used to treat illnesses, including alcoholism and opioid

addiction (Koob and Le Moal, 2006). After watching a friend suffer from cocaine-induced psychosis (characterized by hallucinations that white snakes were slithering over his body and that insects were crawling underneath his skin) in an attempt to use cocaine to alleviate nerve pain (Petersen, 1977), Freud recanted his previous statements on cocaine and published *Craving for and Fear of Cocaine* in 1884 (Koob and Le Moal, 2006).

Cocaine's mechanism of action in the brain is to block monoamine transporters. It has a high affinity for the DA transporter (DAT) in particular, although it also binds to the norepinepherine and serotonin transporters, but with less potency. Cocaine binds to the DAT, blocking the ability of the transporter to remove excess DA from the synaptic cleft, resulting in elevated levels of extracellular DA. This increase in DA is responsible for the euphoric effects reported by cocaine users (Volkow et al., 1999). The excess DA can then bind to more available DA receptors, triggering intracellular cascades that lead to changes in cell firing rates and possible structural changes. Additionally, cocaine blocks sodium channels (Laposata, 1991), which allows the drug to further interfere with the propagation of action potentials.

Cocaine is a stimulant that causes hyperactivity, increased blood pressure, heart rate, and euphoria. The half-life of cocaine is 40-50 minutes, with its euphoric effects peaking at about 15-20 minutes. Excessive doses of cocaine can cause hallucinations, paranoia, seizures, and heart-failure (Withers et al., 1995).

1.5.1 Cocaine-Induced Dopamine, Acetylcholine, and Glutamate Neurotransmission in the Nucleus Accumbens

Cocaine affects a number of major neurotransmitter systems in the brain, including, but not limited to, DA, ACh and glutamate. Cocaine increases DA levels in the brain by blocking DAT, which serves to re-uptake DA into the presynaptic terminal,

thereby leaving more available DA in the synaptic cleft. Dr. Volkow and colleagues have demonstrated, using positron emission tomography (PET), that at least 47% of DAT sites have to be blocked for human subjects to perceive the euphoric effects of cocaine (Volkow et al., 1997). Furthermore, a series of experiments have reported that animals lacking the gene for production of the DAT, called DAT knock out animals, do not exhibit enhanced locomotor activity (Giros et al., 1996), but continue to self-administer cocaine (Rocha et al., 1998) and exhibit conditioned place preference (Sora et al., 1998). This suggests that increased extracellular levels of DA are maintained in the NAcc following cocaine administration even in the absence of this particular monoamine transporter. Carboni et al., (2001) reported that DAT knock out animals did in fact undergo increases in DA levels in the medial NAcc, thereby supporting the role of the NAcc in cocaine reinforcement.

Acetylcholine has been implicated in cognitive processes (Robbins et al., 1997), memory (Torres and Raz, 1994), and also drug abuse and dependency. Rats with an extensive cocaine drug history show enhanced ACh release in the NAcc during cocaine self-administration (Mark et al., 1999). Interestingly, inhibition of acetylcholinesterase (AChE), an enzyme that breaks down ACh, blocks the induction of cocaine-induced hyperactivity as well as prevents the behavioral expression of conditioned place preference in animals receiving repeated i.p. injections of cocaine or morphine (Hikida et al., 2003). These studies suggest that the route of administration and cues associated with drug delivery likely influence the release and overall function of ACh in the NAcc. Moreover, ACh receptors contribute to processes underlying reward and reinforcement. Specifically, muscarinic receptors, such as the m5 receptor expressed on neurons in the VTA, mediate hypothalamic brain stimulation (Yeomans et al., 2000); whereas inactivation of nicotinic receptors, with i.p. administered nicotinic antagonist

mecamylamine, decreases sensitivity to cocaine, evident by disruption of cocaine-induced conditioned place preference (Zachariou et al., 2001). Moreover, both receptor types regulate DA release in the striatum (Zhou et al., 2001; Zhang et al., 2002). Blockade of nicotinic or muscarinic receptors reduces the potency of cocaine (Levin et al., 2000; Rasmussen et al., 2000). Finally, ablation of cholinergic neurons in the NAcc produces increased sensitivity to cocaine by inducing robust conditioned place preference to lower doses of cocaine (Hikida et al., 2001). Such evidence strongly suggests that ACh may also participate in cellular processes underlying cocaine abuse and dependency.

Finally, cocaine increases glutamate release in the NAcc (Smith et al., 1995). A major neuroanatomical circuit recruiting the glutamatergic systems in drug reinforcement is the mesocortical dopaminergic pathway (Pierce and Kalivas, 1997). At the molecular level, cocaine increases DA levels within the PFC and NAcc, receiving dopaminergic input from the VTA (Tzschentke, 2001). Dopamine release in the prefrontal cortex can either activate (Nasif et al., 2005) or inhibit (Peterson et al., 1990) pyramidal neurons whose axon terminals innervate the NAcc. Dopamine D1 receptor activation on pyramidal neurons results in depolarization of these neurons and subsequent release of glutamate in the NAcc. Pharmacological studies show that the glutamatergic increases in the NAcc are heavily dependent on dopaminergic systems. If DA terminals in the NAcc are destroyed or the chemical actions of DA are blocked, cocaine-stimulated glutamate release in the NAcc is also blocked (Reid et al., 1997). At the behavioral level, DA and glutamate release into the NAcc appear to correlate with the expression of behavioral sensitization (Reid and Berger, 1996). Moreover, if NMDA receptors are blocked in the VTA during pretreatment with cocaine, not only is the induction of behavioral sensitization blocked (Kalivas and Alesdatter, 1993), but also neuroadaptations associated with cocaine-induced sensitization, such as DA autoreceptor subsensitivity (Li

et al., 1999b). However, if animals are administered NMDA receptor antagonists after cocaine pretreatment, but before the challenge dose, behavioral sensitization is still observed (Li et al., 1999a). Therefore, NMDA receptors, although not necessary for the expression of cocaine-sensitization, may play a role in the induction of this drug-induced behavioral response.

1.5.2 Cocaine-Induced Molecular Changes and Their Ability to Stimulate Morphological Changes in the Nucleus Accumbens

The neuroadaptations that develop following cocaine administration occur at the molecular as well as the cellular level. At the molecular level, for example, repeated or persistent activation of intracellular cascades can lead to altered receptor sensitivity and synaptic plasticity, which can thereafter influence cellular responses to a repeated stimulus. In fact, a single injection of cocaine can induce long-term potentiation in VTA DA neurons (Borgland et al., 2004). Such molecular switches can cause neuroadaptations, not only in the electrophysiology of cells, but also in cellular morphology. For example, cocaine is reported to affect cytoskeletal proteins, such as Ankyrin (Hayashi and Su, 2001), a protein that modulates calcium efflux from the endoplasmic reticulum and Arc (Tan et al., 2000), a protein expressed in the dendrites of cells. Cytoskeletal proteins are important for cellular functions such as protein trafficking, dendritic growth and morphological maintenance of neurons. Thus, more recent drug studies have been aimed at looking for such morphological changes following long-term drug use. Increases in spine density on the dendrites of MSNs in the NAcc shell (Robinson and Kolb, 1999b) and core (Norrholm et al., 2003) are observed following long-term cocaine treatment; these spine changes appear to be at least in part dependent on the activity of cdk5, a protein that regulates the growth of neurites (Dhavan and Tsai, 2001). These studies demonstrate that cells will change their shape, as a result

of long-term treatment with cocaine. Such evidence further begs the question, are synaptic changes (i.e. changes in the physical connections between brain cells) also taking place?

1.5.3 Cocaine-Induced Locomotor Behaviors and Stereotypies: Use in Studying Sensitization and Tolerance

Cocaine induces a wide range of behaviors in rats, such as hyperlocomotion, excessive exploratory behavior, focused sniffing, headbobbing, as well as other repetitive purposeless behaviors. These repetitive behaviors are called stereotypies (Randrup and Munkvad, 1969), and become more pronounced with long-term drug treatment or with the administration of high drug doses. Drug-induced behaviors change over the course of drug treatment, when rats are given the same drug dose repeatedly over time. Initially rats exhibit hyperlocomotion following cocaine administration, but over time, rats start to spend less time exploring their environment and more time engaged in repetitive, seemingly purposeless behaviors, such as headbobbing and sniffing intensely in one location (Lyon and Robbins, 1975).

Both hyperlocomotion and stereotypies are used to measure persistent behavioral changes as a result of drug treatment. One well-known drug-induced behavior is sensitization. Sensitization is defined as a physiological state characterized by an augmented effect of the drug that occurs following repeated administration of the same drug dose (Kalivas and Duffy, 1990; Robinson and Berridge, 1993). Behavioral sensitization is observed in rats following a single cocaine injection (Guan et al., 1985). Furthermore, the expression of cocaine-induced behavioral sensitization has been linked with increases in Fos expression in the NAcc shell and core (Crombag et al., 2002) as well as an increase in spine density on MSNs, specifically in the NAcc core (Li et al., 2004). Additionally, stereotyped motor behaviors are induced by stimulation of DA

release in the striatum (Fog, 1972) and are abolished by destruction of DA terminals within the striatum (Fibiger et al., 1973). Moreover, these particular behaviors are strongly correlated with Fos activation in the striosomal compartments of the dorsal striatum in the rat (Canales and Graybiel, 2000) and appear to be dependent on the co-activation of D1 and D2 receptors (Capper-Loup et al., 2002), presumably because of their synergistic properties upon concurrent activation. Glutamatergic systems are also implicated in the modulation of cocaine sensitization. Whether glutamatergic systems mediate the development or expression of stereotypic behaviors remains to be determined. At present, DA mechanisms alone do not fully explain the locomotor or stereotypic behaviors that emerge with repeated drug administration (Rebec, 2006). Despite evidence that cocaine does not directly activate glutamate receptors, pretreatment with NMDA or AMPA receptor antagonists prevents the induction of locomotor sensitization to cocaine in drug-naïve animals. Repeated stimulation of the PFC leads to the expression of sensitization (Schenk and Snow, 1994), whereas lesions to the PFC block the development of sensitization (Tzschentke and Schmidt, 1998).

Lastly, behavioral tolerance depends on dose, duration, and frequency of administration. Tolerance to cocaine is believed to be one of the reasons behind dose escalation in chronic users. Cocaine tolerance does not appear to be due to changes in drug metabolism (Inada et al., 1992; Katz et al., 1993) or a change in cocaine levels in the brain (i.e. pharmacokinetic tolerance) following repeated treatment with cocaine (Reith et al., 1987). Supersensitivity of DA D2 autoreceptors is believed to regulate tolerance to cocaine (Ellinwood et al., 2000). In rats receiving cocaine continuously, cocaine-induced locomotor activity can be used to measure motor tolerance (King et al., 1999), yet recovery of weight loss as a result of cocaine treatment is another reported measure for tolerance (Epstein and Altshuler, 1978). To date, cocaine-induced tolerance in rats has

not been correlated with a reduction in the expression of stereotyped behaviors following repeated drug administration. However, stereotyped headbobbing has been correlated with tolerance in rabbits when cocaine is administered prenatally (Simansky and Kachelries, 1996; Stanwood and Levitt, 2003). Stereotyped headbobbing to psychostimulants administration is more pronounced in rabbits; thus, perhaps studies comparing neurochemical changes in rats and rabbits exhibiting tolerance to cocaine can help identify specific neuronal substrates underlying this behavioral adaptation.

1.6 Morphine

Morphine is a natural alkaloid of opium, which comes from the milky juice of the unripe seed of the opium poppy. This alkaloid was named after the Greek god of dreams, Morpheus (van Ree et al., 1999). This chemical binds to endogenous opioid receptors (μ, κ and δ) in the brain, and mice will self-administer morphine directly into the NAcc (Olds, 1982). In fact, mice, if given a choice between the amygdala and VTA, will preferentially self-administer morphine into the VTA (David and Cazala, 1994). Moreover, repeated infusions of morphine into the VTA do not result in the behavioral expression of physical dependence (Bozarth and Wise, 1984). Therefore, morphine's molecular actions in the VTA may be more involved in the rewarding and reinforcing properties of the drug.

Morphine's mechanism of action on the mesolimbic DA pathway involves binding to μ- and δ-opioid receptors expressed by GABAergic (inhibitory) interneurons in the VTA. Activation of this receptor results in hyperpolarization (inhibition of cell firing) of GABA interneurons, thereby activating DA neurons through disinhibition (Gysling and Wang, 1983; Johnson and North, 1992). These molecular events lead to the release of DA in the NAcc, PFC, as well as other limbic-associated brain areas.

Morphine is used medically as an analgesic. However, tolerance quickly develops, requiring higher doses of the drug to control pain. Side effects of long-term opioid use include insomnia, calmness and euphoria followed by dysphoria, increased anxiety, irritability and depression (Barbour and Lilly, 1976; Staedt et al., 1996) as well as a high potential for addiction. Morphine-dependent individuals and animals exhibit physical withdrawal symptoms when drug administration ceases (Himmelsbach, 1942). Tolerance to morphine (or other opioid-derived drugs) increases the likelihood of a drug overdose, which can lead to a comatose state or accidental death (Ellenhorn and Barceloux, 1988).

1.6.1 Morphine-Induced Dopamine, Acetylcholine, and Glutamate Neurotransmission in the Nucleus Accumbens

As previously mentioned, morphine acts on μ- and δ-opioid receptors to indirectly activate DA neurons in the VTA thereby increasing DA release in limbic-related target structures, namely the NAcc. Activation of μ-opioid receptors in the VTA increases DA neurotransmission in the NAcc (Spanagel et al., 1992), whereas activation of κ-opioid receptors decreases accumbal DA release (Di Chiara and Imperato, 1988). Additionally, if morphine is administered directly into the NAcc, the mechanism underlying enhanced DA release appears to be dependent upon the activation of δ-opioid receptors, as opposed to μ-opioid receptors (Borg and Taylor, 1997). Interestingly, robust conditioned place preference is observed in mice that are unable to synthesize DA, thus demonstrating that DA is not required to induce morphine conditioned place preference. Moreover, these animals exhibit a small DA-independent increase in locomotion following morphine administration (Hnasko et al., 2005). Dopamine increases in the NAcc of morphine-sensitized rats are regionally specific. The core compartment of the NAcc shows

increases in DA, while the shell shows decreases relative to control rats (Cadoni and Di Chiara, 1999).

Acetylcholine release in the NAcc has also been reported following the administration of morphine. Acetylcholine levels in the NAcc core and shell decrease following acute morphine administration, but increase after chronic treatment with morphine (Fiserova et al., 1999). The long-lasting changes in accumbal ACh release appear to be regulated by δ-opioid receptors (Tjon et al., 1995). Destruction of ACh-containing neurons in the NAcc results in enhanced sensitivity to morphine's rewarding effects, measured using conditioned place preference; however, in the same study, conditioned place aversion was also increased in these animals during morphine withdrawal (Hikida et al., 2003). In addition, when the metabolism of ACh is blocked, using AChE inhibitors, morphine conditioned place preference is also blocked (Hikida et al., 2003).

The administration of opioids inhibits glutamate release in the NAcc (Sepulveda et al., 1998). Withdrawal from morphine, on the other hand, coincides with an increase in glutamate release in the NAcc (Sepulveda et al., 2004). A more recent study reported a similar increase in glutamate neurotransmission, specifically in the NAcc core, 3 weeks after a single morphine injection (Jacobs et al., 2005). Glutamatergic mechanisms are also thought to mediate behavioral sensitization to morphine. For example, the induction of behavioral motor sensitization is prevented when NMDA or AMPA receptor antagonists are co-administered with morphine (Wolf and Jeziorski, 1993; Jeziorski et al., 1994; Carlezon et al., 1999).

1.6.2 Morphine-Induced Molecular Changes and Their Ability to Stimulate Morphological Changes in the Nucleus Accumbens

Neuroadaptations resulting from chronic opioid administration have been the focus of many recent studies. The NAcc shell and core of morphine-sensitized rats are more metabolically active than drug-naïve animals (Kraus and Kornetsky, 2000). Long-term treatment with morphine, followed by a 1 month withdrawal period, results in a significant decrease in the number of dendritic branches and spine density of MSNs in the NAcc shell (Robinson and Kolb, 1999a; Robinson et al., 2002). One possible mechanism underlying these observed changes in spine density is the ability of μ-opioid receptors to regulate stability of dendritic spines (Liao et al., 2005). Thus, while δ-opioid receptors play more of a role in DA neuron stimulation in the VTA, the μ-opioid receptors potentially affect the integration of glutamatergic and dopaminergic signals, which reportedly converge onto dendritic spines, in the NAcc. The functional relevance of these morphological changes at the behavioral level is still unclear. Recently, cdk5, one of the proteins reportedly responsible for the dendritic spine proliferation resulting from chronic cocaine treatment, has also been implicated as a key molecular substrate involved in the mechanisms underlying morphine-induced behavioral sensitization in mice (Narita et al., 2005) and antinociceptive tolerance in rats (Wang et al., 2004).

1.6.3 Morphine-Induced Locomotor Behaviors and Stereotypies: Use in Studying Sensitization and Tolerance

The behavioral effects observed following acute and chronic morphine administration are described as biphasic, and contrast sharply with those induced by psychostimulants. Low doses of morphine administered to drug-naïve animals produce an inhibitory effect on locomotor activity, called opiate catalepsy (Muley et al., 1982), an effect that can be induced by intracranial infusions of morphine (5-15 μg) directly into the NAcc (Winkler et al., 1982). Moreover, high doses of morphine also produce

hyperlocomotion (Narita et al., 1993; Manzanedo et al., 1999). Dosing and frequency of drug administration, among other variables, greatly impact the expression of drug-induced behaviors. Morphine-induced hyperactivity is regulated to some degree by the actions of DA via the mesolimbic DA pathway. In fact, the VTA, and not the NAcc, appears to be responsible for the hyperactivity observed in morphine-sensitized animals (Kalivas and Duffy, 1987; Vezina et al., 1987). Despite this overlap in neurocircuitry between morphine and psychostimulants (i.e. activation of the mesolimbic DA pathway), morphine-induced hyperactivity is behaviorally different than the hyperlocomotor response to cocaine. First, morphine-induced hyperactivity is limited to increases in lateral activity and sniffing behavior, none of which is correlated with an increase in rearing activity (Laviola et al., 1994; Kuzmin et al., 2000). Second, unlike cocaine-induced hyperlocomotion, DA receptors do not appear to play a major role in mediating opioid sensitization (Kalivas et al., 1985; Jeziorski and White, 1995). Thus the cellular mechanisms underlying morphine sensitization require further examination. Cross-sensitization studies will likely prove useful tools for differentiating the mechanisms underlying such persistent behavioral adaptations.

Stereotyped behaviors due to chronic morphine treatment include excessive chewing/gnawing (Pollock and Kornetsky, 1989) and licking (Cadoni and Di Chiara, 1999). The shell and core compartments of the NAcc show enhanced metabolic activity in morphine-sensitized animals exhibiting this oral stereotypy (Kraus and Kornetsky, 2000). The precise neuronal mechanism responsible for this behavior is poorly understood. Fluoxetine (a serotonin reuptake inhibitor), MK-801 (NMDA receptor antagonist), and SCH23390 (DA D1 receptor antagonist) all block the expression of this morphine-induced oral stereotypy; however, only the NMDA receptor antagonist

partially prevented the development of sensitization (Livezey et al., 1995; Wennemer and Kornetsky, 1999).

Morphine-induced locomotor activity as well as stereotyped chewing/gnawing has been used to measure opioid-induced sensitization, while tolerance to morphine is typically measured by its reduced analgesic effects over time. Tolerance occurs quickly and is more widely reported in morphine-related studies, as compared to studies involving psychostimulants. Opioid tolerance to pain is typically measured using the tail flick response (Grumbach and Chernov, 1965), which measures the diminishing analgesic effects of morphine during the course of repeated administration (Patrick et al., 1978; Siegel et al., 1981). Less well-known, and less well-studied, is opioid catalepsy (De Ryck et al., 1980; De Ryck and Teitelbaum, 1984; Fischer et al., 2002) as a measure of motor tolerance in the rat (Walter and Kuschinsky, 1989).

Chapter 2: Cholinergic Interneurons of the Nucleus Accumbens and Dorsal Striatum are Activated by the Self-Administration of Cocaine

2.1 Abstract

The nucleus accumbens, a major component of the ventral striatum, and the dorsal striatum are primary targets of the mesolimbic dopamine pathway, which is a pathway that plays a critical role in reward and addiction. The shell compartment of the nucleus accumbens and the ventromedial striatum, in particular, receive extensive afferent projections from the ventral tegmental area, which is the major afferent source of the mesolimbic pathway (Gerfen et al., 1987; Amalric and Koob, 1993). The present study tested the hypothesis that striatal cholinergic interneurons of the nucleus accumbens are activated by the acute self-administration of cocaine. The main finding of this study is that cholinergic interneurons located in the dorsal medial shell compartment of the nucleus accumbens and the ventromedial striatum were activated, as measured by Fos labeling, following a 1 h session of the self-administration of cocaine in rats. A direct positive correlation existed between the percent of cholinergic interneurons that were activated and the amount of cocaine that was self-administered. The greatest amount of administered cocaine (approximately 10 mg/kg) resulted in the activation of approximately 80% of the cholinergic neurons. No such correlation existed in the group of animals that self-administered saline. In addition, activation was not found in the core compartment of the nucleus accumbens or the dorsolateral striatum, which receive extensive innervation from the substantia nigra and thus are more closely tied to the motor effects of the drug. In conclusion, cocaine-driven neuronal activation was specific to the shell compartment of the nucleus accumbens (R^2=0.9365) and the ventromedial striatum (R^2=0.9059). These findings demonstrate that cholinergic interneurons are

involved in the initial stage of cocaine intake and that these neurons are located in areas of the nucleus accumbens and dorsal striatum that are more closely tied to the rewarding and hedonic effects rather than the motor effects of cocaine intake.

2.2 Introduction

The nucleus accumbens (NAcc) and the dorsal striatum have traditionally been regarded as an interface between limbic and motor systems. These areas of the brain receive extensive dopaminergic innervation from the ventral tegmental area (VTA) and the substantia nigra (SN), which subserve primarily limbic and motor function, respectively. The mesolimbic DA pathway originating in the VTA, which is targeted by several drugs of abuse including cocaine, amphetamine, heroin, morphine, nicotine, and alcohol (Self and Nestler, 1995; Berke and Hyman, 2000; Koob and Le Moal, 2001; Nestler, 2001), extensively innervates the shell compartment of the NAcc and the ventromedial striatum (Gerfen et al., 1987; Amalric and Koob, 1993). The SN, which is primarily involved in motor function, instead primarily innervates the core region of the NAcc (Nirenberg et al., 1996) and the dorsolateral striatum (Gerfen and Sawchenko, 1984). In order to understand the behavioral correlates and cellular adaptations that accompany addiction, it is essential to examine these striatal pathways both at the global systems level as well as at the cellular level. The neostriatum, therefore, should be examined in terms of its functionally distinct components, at the level of individual neurons and related microcircuitry, in order to fully understand the cellular and behavioral mechanisms that underlie drug abuse and addiction.

The focus of the present study was on a specific cell type, striatal cholinergic interneurons, which are important neuronal integrators and modulators of striatal function and dysfunction. These neurons possess receptors that are linked to molecular signaling pathways, which are critical for plasticity (Calabresi et al., 1999), long-term potentiation

(LTP) (Suzuki et al., 2001), and associative learning (Aosaki et al., 1994). Cholinergic interneurons in turn exert powerful influences on medium spiny output neurons (MSNs) and thus on overall striatal signaling (Howe and Surmeier, 1995). Cholinergic neuromodulation specifically facilitates LTP expression in MSNs as well as N-methyl-D-aspartate (NMDA) receptor-mediated corticostriatal signaling onto MSNs (Calabresi et al., 1992; Calabresi et al., 2000). Accumulating evidence from pharmacological and behavioral studies further suggests a central role for cholinergic neurons in mediating addiction-related behaviors. Pharmacological evidence, for example, has shown that acetylcholine (ACh) levels increase in the NAcc following the administration of cocaine in the rat (Consolo et al., 1999; Mark et al., 1999).

The present study tested the hypothesis that cholinergic interneurons in specific areas of the NAcc (shell) and the dorsal striatum (ventromedial striatum), which are targeted by the mesolimbic DA pathway, are activated by the self-administration of cocaine. Together, the findings implicate cholinergic interneurons in these areas of the neostriatum in addictive processes. (This study has been published: Berlanga et al., 2003).

2.3 Methods

Each experimental group (Acute or Chronic) was divided further into those receiving cocaine or saline. Animals were allowed to self-administer during a single 1-h session (Acute group) or for daily 1-h sessions across a 2-week period (Chronic group). Rats were anesthetized and perfused 2 h following the end of the last self-administration session.

2.3.1 Animals

Thirty-two male Sprague–Dawley rats were obtained from the Animal Resource Center at the University of Texas at Austin. All experimental procedures conformed to National Institutes of Health guidelines and were carried out under an institutionally reviewed and approved research protocol. Animals were group housed and handled for 2 weeks, and then trained to lever press for sugar pellets on a fixed ratio 1 (FR1) schedule for 1 week before receiving surgery. All efforts were made to minimize animal suffering and the number of animals used.

2.3.2 Surgical Procedures

Rats were anesthetized with sodium pentobarbital (50 mg/kg, i.p.) and supplemented with chloral hydrate (80 mg/kg, i.p.) as needed. Atropine sulfate (350 μg/animal) was administered prophylactically to alleviate potential respiratory congestion. A silastic catheter (0.625 mm, o.d.) was inserted into the right external jugular vein. The free end of the catheter, fused with a modified cannula termination (C313G; Plastics One, Roanoke, VA, USA), was run subcutaneously along the side of the neck and out an incision in the skin at the top of the skull. The cannula termination was embedded into dental acrylic and secured to the skull with four stainless steel screws. Intravenous catheter patency was maintained as previously described (Emmett-Oglesby et al., 1993).

2.3.3 Self-Administration

Self-administration procedures were conducted as previously described (Ikegami et al., 2002). Briefly, self-administration sessions were 1 h in duration. Saline and cocaine injections were infused according to an FR1 schedule followed by a 20-s timeout. Each lever press resulted in an infusion of 0.75 mg/kg or an equal volume of saline and

illumination of a stimulus light. Locomotor activity was assessed by photobeam breakage at three locations along the length of the operant chambers. Experimental programs were run and data were acquired using an IBM compatible computer using MED-PC software (MED Assoc., St. Albans, VT, USA).

2.3.4 Tissue Preparation

Animals were anesthetized deeply with sodium pentobarbital and perfused transcardially with 50 ml of 0.1 M phosphate-buffered saline (PBS) followed by 200 ml of 4% paraformaldehyde/0.1% glutaraldehyde (pH 7.4). Brains were removed and post-fixed for 2 h. Vibratome sections (100 μm thick) were collected into 0.1 M PBS, and frozen in liquid nitrogen and stored at −80 °C until they were processed for immunocytochemical labeling.

2.3.5 Dual Immunocytochemistry

Free-floating sections were washed in 0.1 M PBS and washed again in PBS following each of the subsequent steps. Sections were incubated in 5% normal goat serum (NGS)/1% H_2O_2 in PBS for 1 h. Sections were then incubated in a cocktail of primary antibodies for choline acetyl-transferase (ChAT; monoclonal, 1:1000; Chemicon, Temecula, CA, USA) and c-Fos (1:1000; Santa Cruz Biotechnology, Santa Cruz, CA, USA) in 5% NGS overnight at 4 °C. The tissue was then incubated in secondary biotinylated donkey anti-mouse IgG antiserum (1:500; Jackson ImmunoResearch, West Grove, PA, USA), diluted in 2% NGS-PBS for 2 h, and then incubated in an avidin–biotin peroxidase complex (ABC; Vectastain Elite Kit; Vector, Burlingame, CA, USA) for 1 h. ChAT-immunoreactivity (IR) was visualized with 0.05% 3,3′-diaminobenzidine tetrahydrochloride (DAB) and 0.01% H_2O_2 in PBS which resulted in a brown reaction product. The sections were then incubated for 1 h in biotinylated donkey anti-rabbit IgG

(1:200; Chemicon, Temecula, CA, USA) followed by incubation in ABC for 1 h. Fos-IR was then visualized with a Vector SG (blue) label.

2.3.6 Quantitative Analysis

Three digital images representing areas of 0.45 mm2 for each hemisphere along the dorso-ventral extent of the medial NAcc shell and core, and one digital image representing an area of 0.45 mm2 for each hemisphere in the ventromedial and dorsolateral striatum were acquired from tissue from each animal using a 10× objective on a Nikon Eclipse E800 light microscope. Images were stored and analyzed by two observers blind to group assignment. The observers counted all cholinergic cells in the 0.45 mm2 sample area (as determined by brown DAB label) and determined the percent of cholinergic cells that co-expressed Fos as indicated by the blue SG label. The total percent of Fos-labeled cholinergic neurons per animal was calculated as an average of the total sampled areas. The areas sampled were taken from the medial quadrant of the shell compartment of the NAcc and the ventromedial striatum (1.60 mm Bregma (Paxinos and Watson, 1998)), which is an area that receives heavy dopaminergic innervation from the VTA. In addition, samples were also taken from two areas associated with motor function, the core compartment of the NAcc and the dorsolateral striatum. These two areas receive their dopaminergic innervation primarily from the SN.

2.4 Results

Cholinergic interneurons of the dorsal and ventral striatum of the rat were identified using immunocytochemistry procedures and an antibody against ChAT. Fig. 2.1A illustrates ChAT-immunolabeled neurons of the striatum. Cholinergic cells that were activated were determined by dual immunolabeling for ChAT and Fos protein. A representative photomicrograph of Fos-labeled cholinergic cells is shown in Fig. 2.1B.

The percent of neurons activated were calculated from samples taken from specific regions of the dorsal and ventral striatum (1.60 mm Bregma(Paxinos and Watson, 1998)) that are innervated by the VTA and SN (Fig. 2.2).

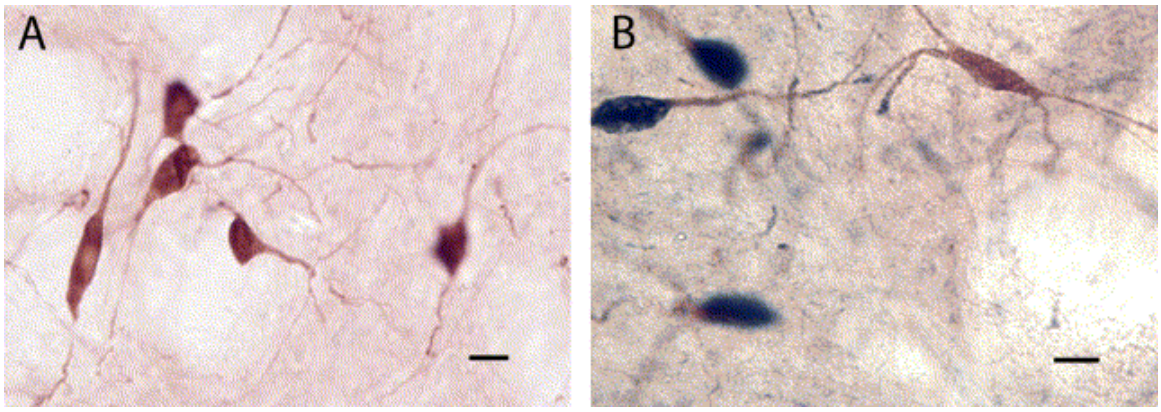

Figure 2.1 Fos immunoreactive cholinergic interneurons. Cholinergic interneurons expressing Fos located in the striatum of the rat brain were identified by immunocytochemical dual labeling procedures using an anti-ChAT antibody and DAB label (brown) and an anti-Fos antibody and SG label (blue). (A) An image illustrating cholinergic interneurons identified by ChAT immunolabeling. Cholinergic cell somata measured 30–50 μm in diameter and their neuronal processes measured up to a millimeter in length. The neurons were frequently situated in neuronal clusters. (B) Tissue dual labeled for ChAT and Fos identified cholinergic interneurons (brown) and cholinergic interneurons that were Fos-positive (brown/blue). Scale bars =30 μm.

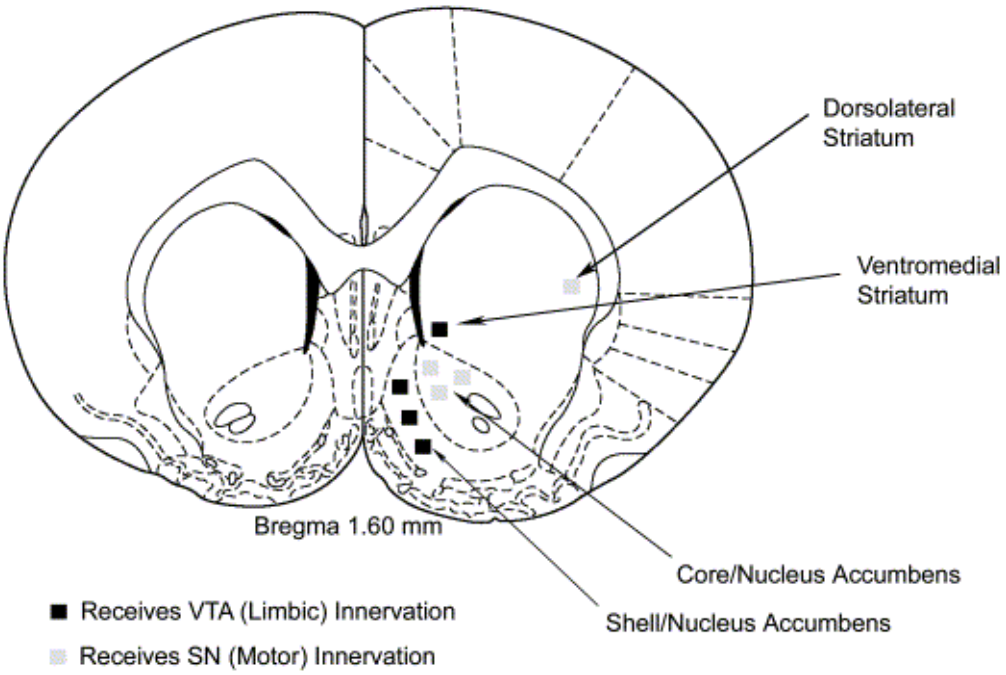

Figure 2.2 Nucleus accumbens and striatum atlas plate used for quantification. A Coronal section of the rat brain at Bregma 1.60 mm (Paxinos and Watson, 1998) showing regions of the NAcc and dorsal striatum where photomicrographs were taken and used for quantification procedures (represented by black and gray boxes). Cholinergic interneurons in the shell compartment of the NAcc and the ventromedial striatum are areas that are innervated by the VTA (represented by black boxes). These areas revealed a direct correlation between Fos expression and the self-administration of cocaine. However, cholinergic cells in areas innervated by the SN, namely the core compartment of the NAcc and the dorsolateral striatum (represented by gray boxes) did not show a direct correlation between Fos expression and the self-administration of cocaine.

The amount of cholinergic neuronal activation that occurred, as measured by Fos labeling, was strongly correlated with the amount of cocaine that was self-administered. In other words, a greater percentage of activated cholinergic neurons located in the shell compartment of the NAcc and the ventromedial striatum occurred with increased levels of the self-administration of cocaine compared with the self-administration of saline. The greatest amount of cocaine that was self-administered (approximately 10 mg/kg) resulted

in the activation of 84% of the cells in the shell compartment of the NAcc and 79% of the cells in the ventromedial striatum. No such correlation existed in the group of animals that self-administered saline. Cocaine-driven neuronal activation was specific to the shell compartment of the NAcc (R^2=0.9365) and the ventromedial striatum (R^2=0.9059). Neuronal activation in these areas did not increase with increased levels of saline administration in either the NAcc (R^2=0.2307) or the ventromedial striatum (R^2=6E-05). Furthermore, activation was not found in the motor-associated core compartment of the NAcc in the cocaine (R^2=0.0413) or saline (R^2=0.0961) group or in the dorsolateral striatum in the cocaine (R^2=0.0772) or saline group (R^2=0.2456; Fig. 2.3). In addition, cholinergic activation did not appear to be due to motor activity, since neuronal activation was not correlated with the amount of bar pressing that occurred in the saline group (data not shown). Therefore, the greatest amount of cholinergic neuronal activation was not associated with saline administration or with motor activity per se. Instead, neuronal activation was specifically associated with the self-administration of cocaine.

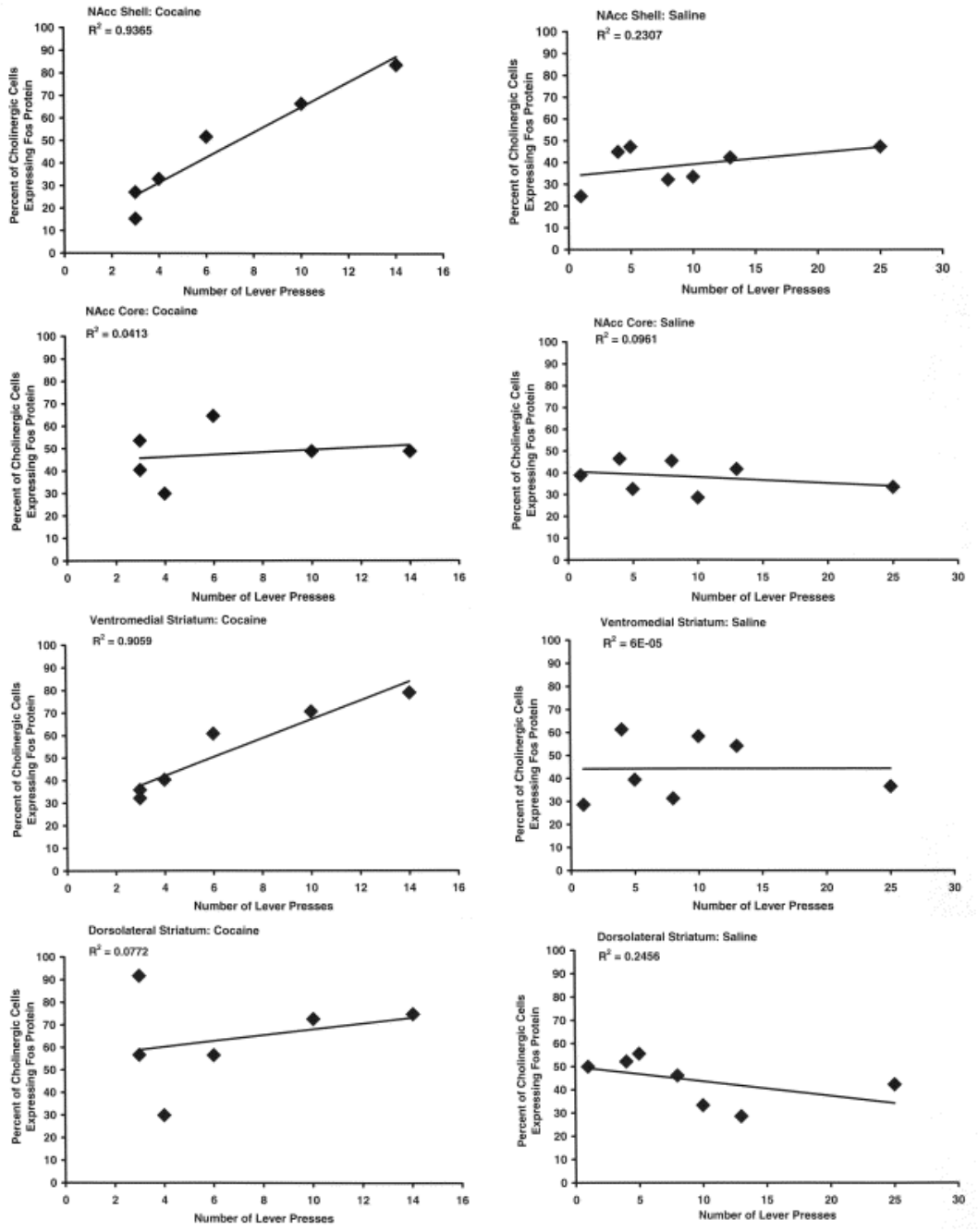

Figure 2.3 Percent of Fos immunoreactive cholinergic cells following cocaine self-administration. The percent of cholinergic cells that were activated, as measured by Fos, in the NAcc or dorsal striatum, plotted against the number of lever presses performed by a rat in a 1-h session of the self-administration of

cocaine or saline. Each lever press resulted in an infusion of 0.75 mg/kg of cocaine or saline. A direct correlation existed between the percent of Fos-IR (activated) cholinergic interneurons and the amount of self-administered cocaine in the shell compartment of the NAcc (R^2=0.9365) and the ventromedial striatum (R^2=0.9059), which are areas that receive VTA innervation. No correlation existed for the self-administration of saline in either the NAcc (R^2=0.2307) or the ventromedial striatum (R^2=6E-05). The amount of activated cholinergic neurons and the amount of self-administered cocaine or saline was not correlated in the motor areas of the NAcc, which are targeted by the SN. Specifically, the core compartment of the NAcc (cocaine: R^2=0.0413, saline: R^2=0.0961) and the dorsolateral striatum (cocaine: R^2=0.0772, saline: R^2=0.2456).

2.5 Discussion

The main finding of this study is that the cholinergic interneurons of the NAcc and dorsal striatum were activated in a dose dependent manner following a 1 h session of the self-administration of cocaine in rats. Specifically, the neurons activated were cholinergic interneurons located in the shell compartment of the NAcc and the ventromedial striatum. These brain areas receive direct innervation from the dopaminergic mesolimbic pathway, which plays an important role in reward and addiction (Self and Nestler, 1995).

Cholinergic interneurons of the striatum possess a number of behavior-related, cellular, and molecular characteristics that position them as likely candidates important for mediating reinforcement and addiction-related phenomena. Specifically, cholinergic interneurons of the striatum integrate a variety of cognitive, limbic, and motor information (Calabresi et al., 2000). These neurons further undergo plasticity and learning and in turn influence striatal output signaling (Aosaki et al., 1994). The activation of these cholinergic neurons in the shell NAcc and ventral striatum following the first administration of cocaine suggests that these neurons play a role in the initial stage of drug intake and possibly in the learning mechanisms involved in the first exposure to the drug. The fact that the activation of these neurons was not correlated with

cocaine intake in the motor-associated core NAcc and dorsolateral striatum is consistent with pharmacological data that reports a dissociation between locomotor behavior and striatal ACh release (Zocchi and Pert, 1994). In the present study, we have identified a potential neuronal cell type and related microcircuitry, i.e. the cholinergic neuronal network system that may be critical for the initiation and maintenance of drug reinforcement and addiction. Fig. 2.4 illustrates the key cellular and molecular characteristics of cholinergic interneurons and their positioning within a more global systems level, which, overall, may play an important role in reinforcement and addiction-related processes.

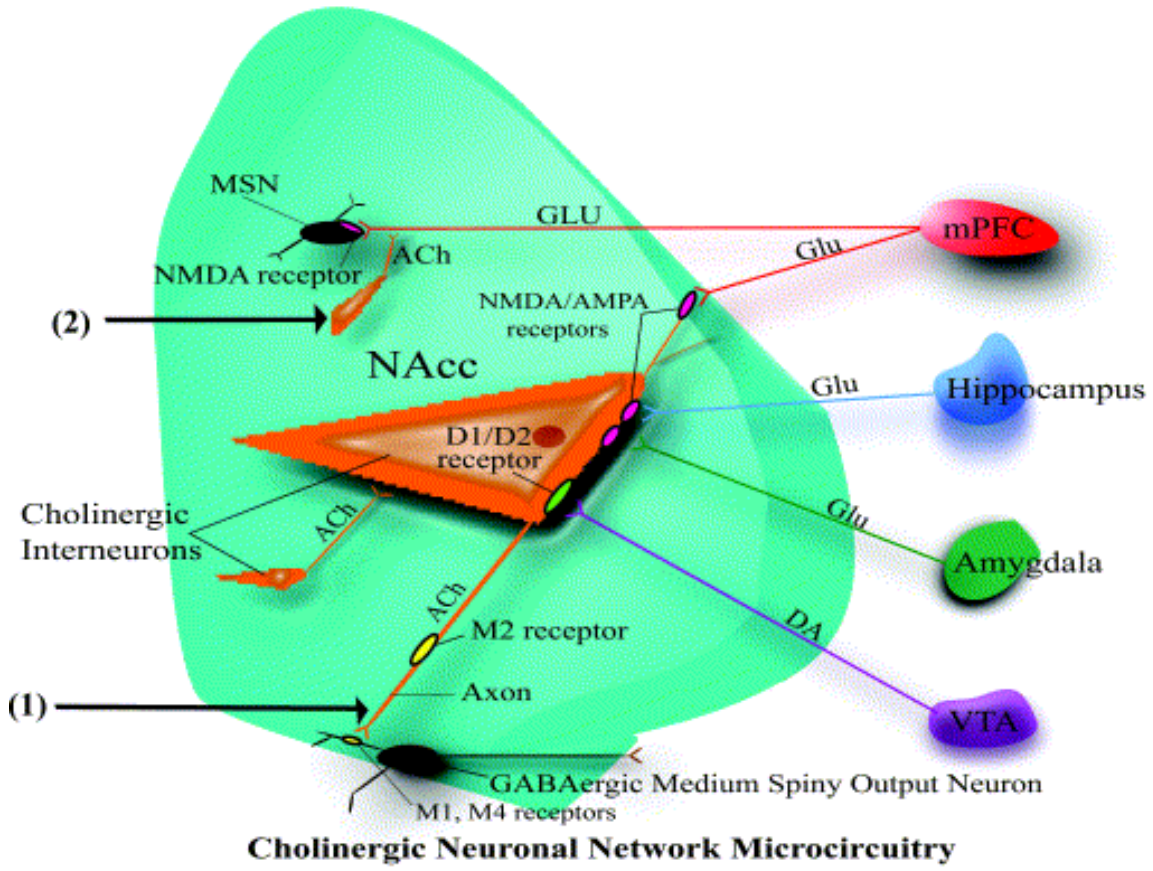

Figure 2.4 Schematic of the cholinergic microcircuitry of the nucleus accumbens. This schematic depicts the cholinergic neuronal network system of the NAcc, in particular the microcircuitry involving dopamine (DA) and glutamate (GLU) afferent connections and receptors. The cholinergic neuronal network system receives and integrates important cortical and subcortical information and in turn provides a powerful influence on MSNs and thus on overall striatal signaling. The DA–GLU convergence onto these cholinergic neurons may provide a neural substrate by which the DA-mediated rewarding or hedonic effects of drugs are associated with such GLU-mediated information as contextual or motivational stimuli. These neurons, therefore, may play an important role in the cellular mechanisms that underlie learning and addiction. Overall, cholinergic neurons are posited to play an important role in (1) the establishment and maintenance of drug addiction via influences onto MSNs and (2) the modulation of incoming prefronto-striatal signaling. The PFC has the capacity to drive drug-seeking behaviors and relapse; however, it may also serve to inhibit striatal circuits that drive compulsive drug-seeking behaviors. Thus, cholinergic interneurons are an important component of the cellular and behavioral mechanisms that may drive addiction, but may also be key to the prevention and treatment of drug abuse and addiction. mPFC, medial prefrontal cortex.

Cholinergic involvement in drug addiction

Increasing evidence from behavioral and pharmacological studies has convincingly demonstrated the importance of ACh in drug addiction. Pharmacological evidence, for example, has shown that ACh levels increase in the NAcc following the acute administration of cocaine (Consolo et al., 1999) and following chronic self-administration of cocaine in rats (Mark et al., 1999). Such cocaine-induced striatal in vivo ACh release is regulated by dopamine (DA) D5 receptors (Consolo et al., 1992), which we have recently localized on cholinergic neurons (Berlanga et al., 2005). In accordance with these findings, we have provided compelling evidence in this paper that cholinergic interneurons of specific regions of the NAcc and dorsal striatum, as demonstrated by Fos labeling, are indeed activated following a 1 h session of the self-administration of cocaine in rats. It has been suggested that transient immediate early gene protein expression in neurons may trigger a cascade of cellular events that underlies long-term information storage, which is modified over time by a myriad of significant behavioral and cognitive stimuli (Clayton, 2000). It is possible, therefore, that although Fos labeling is a transient neuronal marker, that Fos expression may trigger neuroadaptive changes in receptor expression or long-term changes in synaptic wiring in cholinergic neuronal network systems in chronic and withdrawal conditions, which are questions that are currently under investigation in our laboratory.

Pharmacological evidence supports the importance of ACh addiction-related phenomena. Systemic application of ACh muscarinic receptor blockers prevents the induction of behavioral sensitization to cocaine (Heidbreder and Shippenberg, 1996; Karler et al., 1996), suggesting a role for ACh in behavioral sensitization. Withdrawal from a sensitizing regimen of cocaine, on the other hand, produces transient, but dramatic up-regulation of both muscarinic M2 and dopamine D2 receptors (Sousa et al., 1999),

which in turn attenuate the response of ACh release (Calabresi et al., 1999). Interestingly, cocaine has relatively high affinity for the muscarinic M2 receptor, as well as the DA transporter (Sharkey et al., 1988). Given the discrete segregation of muscarinic M2 receptor subtypes (Alcantara et al., 2001), as well as the presence of DA D2 receptors on cholinergic interneurons (Alcantara et al., 2003), it is highly probable that cholinergic interneurons of the NAcc play an important role in the neuroadaptations that accompany not only drug intake, but also withdrawal. Following this study, an analysis of DA D2 and D5 receptor neuroadaptations, associated with cholinergic neuronal populations in the NAcc shell and core, was completed using a subsequent self-administration paradigm involving two drug conditions, chronic (2 weeks) cocaine and withdrawal (2 weeks cocaine followed by two weeks of abstinence). However, due to the low amounts of cocaine that were self-administered by the animals used in the study, the results were inconclusive. Additionally, drugs that enhance cholinergic neurotransmission in the medial prefrontal cortex, an area known to modulate mesoaccumbens DA transmission (Olsen and Duvauchelle, 2001), also increase instrumental responding for cocaine (Ikemoto and Goeders, 2000). Alternatively, lesions of the prefrontal cortex prevent induction of cocaine sensitization (Pierce et al., 1998; Li et al., 1999a). Specific lesions of cholinergic neurons in the nucleus basalis magnocellularis, a target of NAcc innervation, also attenuate the self-administration of cocaine (Robledo et al., 1998).

Cholinergic neurotransmission may serve both to facilitate and attenuate addiction-related phenomena

The specific role that striatal cholinergic interneurons play in drug reinforcement and addiction will likely depend on their positioning within specific neuronal pathways and microcircuits. At the microcircuit level, ACh release will affect a variety of

muscarinic and nicotinic receptor subtypes, which evoke unique intracellular signaling mechanisms and physiological effects. At the systems level, cholinergic function will depend on the positioning of these cells within functionally distinct striosomal or matrix compartments and the direct or indirect striatal output pathways. The cholinergic–cholinergic synaptic connections as well as the large size of cholinergic neurons and their extensive dendritic and axonal arborizations, in particular, may serve to provide a powerful cholinergic neuronal network influence over striatal functioning. The afferent/efferent connections of the cholinergic neurons will also dictate overall ACh function. In addition to the dopaminergic mesolimbic innervation, which has been implicated in incentive and habit learning (Di Chiara, 1998), the NAcc and dorsal striatum are also innervated by glutamatergic cortical and limbic afferents. Such dopamine–glutamate convergent influences onto cholinergic neurons may be especially important for mediating associative learning, which is an important process underlying addiction (Di Chiara, 1999; Vanderschuren and Kalivas, 2000).

Associative learning has been reported to underlie context-specific psychomotor sensitization (Anagnostaras et al., 2002), which involves alterations in dopaminergic and glutamatergic neurotransmission (Vanderschuren and Kalivas, 2000) and may be linked to addiction. Such associative learning may likely involve the convergence of reward-mediated mesolimbic dopaminergic afferents with hippocampal glutamatergic afferents onto MSNs of the NAcc. The MSNs have previously been reported to demonstrate altered plasticity in response to cocaine or amphetamine (Robinson and Kolb, 1999b; Robinson et al., 2001). It will be critical to examine whether such mesolimbic and hippocampal convergence exists onto MSNs, but more importantly, whether such convergence and altered synaptic plasticity occurs within cholinergic microcircuits. Specifically, the cholinergic interneurons of the medial shell compartment of the NAcc

and the ventromedial striatum, which we have shown to be activated by the self-administration of cocaine, and more recently by the acute administration of alcohol (Herring et al., 2004), will be critical to study since these brain areas receive dopaminergic VTA inputs (Newman and Winans, 1980) as well as glutamatergic innervation from the amygdala (Kita and Kitai, 1990) and hippocampus (Kelley and Domesick, 1982).

Remarkably, ACh neurotransmission may play an important role not only in facilitating addiction, but also in the prevention and treatment of addiction. It has recently been reported that ACh facilitates LTP expression in MSNs as well as prefrontal–striatal signaling onto MSNs via muscarinic m1 receptors (Calabresi et al., 1999; Alcantara et al., 2001). The prefrontal cortex exerts an executive function over a variety of cortical and subcortical structures, and may have an important influence in either driving or attenuating compulsive drug seeking behavior and relapse. Therefore, cholinergic interneurons may serve to (1) facilitate the striatal functions that drive addiction and (2) mediate prefrontal–striatal signaling onto MSNs involved in either the facilitation or attenuation of addiction-related behaviors (see Fig. 2.4). It is critical to study and understand the specific microcircuitry and molecular signaling pathways of the cholinergic neuronal network system, especially as they pertain to incoming glutamatergic prefronto-striatal neurotransmission. Furthermore, it is also important to focus on identifying areas that are especially susceptible to plasticity and learning. Finally, because this microcircuitry is located at the interface of the striatum and the prefrontal cortex, which is an area involved in executive function, it may be an important site to focus on when designing improved pharmaceutical and behavioral programs designed for the treatment and prevention of drug abuse and addiction.

Chapter 3: Dopamine D5 Receptor Localization on Cholinergic Neurons of the Rat Forebrain and Diencephalon: A Potential Neuroanatomical Substrate Involved in Mediating Dopaminergic Influences on Acetylcholine Release

3.1 Abstract

The study of dopaminergic influences on acetylcholine release is especially useful for the understanding of a wide range of brain functions and neurological disorders, including schizophrenia, Parkinson's disease, Alzheimer's disease, and drug addiction. These disorders are characterized by a neurochemical imbalance of a variety of neurotransmitter systems, including the dopamine and acetylcholine systems. Dopamine modulates acetylcholine levels in the brain by binding to dopamine receptors located directly on cholinergic cells. The dopamine D5 receptor, a D1-class receptor subtype, potentiates acetylcholine release and has been investigated as a possible substrate underlying a variety of brain functions and clinical disorders. This receptor subtype, therefore, may prove to be a putative target for pharmacotherapeutic strategies and cognitive-behavioral treatments aimed at treating a variety of neurological disorders. The present study tested the hypothesis that cholinergic cells in the dopamine targeted areas of the cerebral cortex, striatum (dorsal striatum and nucleus accumbens), basal forebrain, and diencephalon express the dopamine D5 receptor. These receptors were localized on cholinergic neurons with dual labeling immunoperoxidase or immunofluorescence procedures using antibodies directed against choline acetyltransferase (ChAT) and the dopamine D5 receptor. Results from this study support previous findings indicating that striatal cholinergic interneurons express the dopamine D5 receptor. In addition, cholinergic neurons in other critical brain areas also show dopamine D5 receptor expression. Dopamine D5 receptors were localized on the somata, dendrites, and axons of

cholinergic cells in each of the brain areas examined. These findings support the functional importance of the dopamine D5 receptor in the modulation of acetylcholine release throughout the brain.

3.2 Introduction

Several major cholinergic systems exist in the rat brain, including the cholinergic interneurons of the cerebral cortex (Houser et al., 1985) and the striatum (Butcher and Woolf, 1982; Bolam et al., 1984), as well as the projection neurons arising from the basal forebrain (Zaborszky et al., 1993) and the diencephalon (Manger et al., 2002). Cholinergic neurons in each of these systems form intricate networks necessary for a number of critical functions, including cognition, motivation, learning and memory, and motor functions (Mesulam et al., 1983; Woolf, 1991; Kasa et al., 1997; Pisani et al., 2003). Dopaminergic innervation of these cholinergic brain regions occurs primarily through the dopamine (DA) afferents arising from the mesencephalon, more specifically the ventral tegmental area (VTA) and the substantia nigra (SN) (Swanson, 1982). Furthermore, evidence of dopaminergic modulation of cholinergic neurotransmission has been previously reported (Day and Fibiger, 1992; De Boer et al., 1992; Day and Fibiger, 1993; Imperato et al., 1993). The focus of this study was to localize DA D5 receptors, a DA D1-class receptor subtype, on cholinergic neurons of the cerebral cortex, striatum, basal forebrain, and the diencephalon (see Fig. 3.1), which may serve as potential neural substrates involved in mediating dopaminergic influences on acetylcholine (ACh) release.

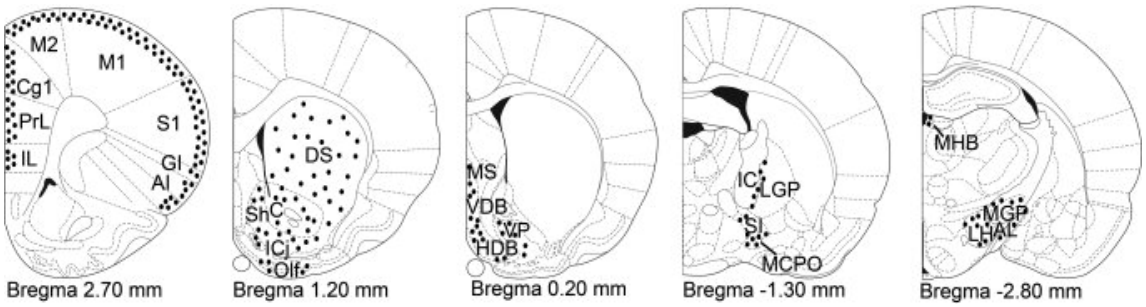

Figure 3.1. Cholinergic cell localization in the rat brain. Schematic coronal sections of the rostral-caudal axis of the rat brain show the regions where DA D5-positive cholinergic neurons (indicated by dots) were found. Cholinergic cells located in the rat forebrain cortices at Bregma 2.70 mm and neostriatum at Bregma 1.20 mm are interneurons, whereas cholinergic cells located in the basal forebrain at Bregma 0.20 mm and -1.30 mm, and in the basal forebrain and diencephalon at Bregma -2.80 mm are projection neurons.

The cerebral cortex of the rat not only receives dense cholinergic innervation from projection neurons of the basal forebrain, but also has an intrinsic source of ACh provided by local cholinergic interneurons, which provide up to 30% of the total cholinergic innervation of the cerebral cortex (Mesulam et al., 1983; Houser et al., 1985). The specific role of these local cortical neurons has not been clearly defined, which may be due to the difficulty in selectively targeting cholinergic cells for analysis in the cortex. Saporin-induced lesions of cholinergic fibers of the cerebral cortex, for example, may target not only intrinsic cholinergic cells, but also cholinergic projections from the basal forebrain (Dalley et al., 2004). The cortex receives extensive mesencephalic dopaminergic innervation (Berger et al., 1974); however, direct evidence of DA synapses onto cortical ACh-containing interneurons has not been reported. It is unclear, therefore, whether DA modulation of ACh release from these cortical neurons occurs through

synaptic or nonsynaptic (i.e., volume transmission) mechanisms or possibly through a combination of both types of mechanisms. The localization of DA D5 receptors on these cells suggests specific receptor-guided mechanisms involved in ACh release from these local circuit neurons. More precise characterization of DA-ACh interactions and the functional role of these ACh-containing neurons in the cerebral cortex, however, remain to be further investigated.

Acetylcholine in the striatum is supplied predominantly from large, intrinsic cholinergic interneurons, which account for ~2% of the entire striatal neuronal population. These neurons and their functions, unlike the cholinergic neurons of cerebral cortex, have been well characterized. Striatal cholinergic neurons have been implicated in such phenomena as long-term potentiation (LTP), synaptic plasticity, and associative learning (Aosaki et al., 1994). These cells have further been reported to modulate LTP expression in striatal projection neurons (Calabresi et al., 1999) and prefronto-striatal information processing (Alcantara et al., 2001) as well as overall striatal signaling (Howe and Surmeier, 1995). Striatal cholinergic interneurons are targeted by several neurotransmitter systems including the DA mesencephalic system. Dimova et al. (1993) reported that striatal cholinergic cells receive direct synaptic contact from tyrosine hydroxylase (TH)-positive nerve terminals. DA receptors located on cholinergic neurons, therefore, may influence ACh release in the striatum through synaptic as well as possible nonsynaptic mechanisms. Further studies have reported the involvement of specific DA receptor subtypes in cholinergic neuronal function. The induction of LTP in striatal cholinergic neurons, for example, has been reported to be DA D5 receptor-dependent (Suzuki et al., 2001). Imbalances in DA-ACh levels in the striatum and other forebrain structures, conversely, have been implicated in such disorders as schizophrenia (Sarter and Bruno, 1998; Holt et al., 1999), Parkinson's disease (Rinne et al., 1989; Pisani et al.,

2003), Alzheimer's disease (Gsell et al., 2004), and addiction (Consolo et al., 1999; Mark et al., 1999; Berlanga et al., 2003; Herring et al., 2004).

The cholinergic projection systems of the basal forebrain and the diencephalon innervate a variety of structures and, as a result, affect a wide variety of functions. The cholinergic neurons of the basal forebrain send dense projections to the cerebral cortex, hippocampus, amygdala, and the olfactory bulb (Zaborszky et al., 1993), mediating such functions as attention, arousal, motivation, and learning (Dekker et al., 1991; Fibiger, 1991; Muir et al., 2001). Similarly, cholinergic cells of the diencephalon, namely, the medial habenular nucleus, project to the interpeduncular nucleus and, in addition to controlling behaviors such as arousal, also regulate sleep cycles and the coordination of limb movement (Sutherland, 1982). Both the basal forebrain and diencephalic cholinergic projection systems receive dopaminergic input from the mesencephalon (Fallon and Moore, 1978; Phillipson and Pycock, 1982; Zaborszky et al., 1993), with the diencephalon receiving only a small percentage of DA from local DA neurons (Eaton et al., 1994). Furthermore, degeneration of the basal forebrain and its related circuitry is pivotal to memory loss observed in such clinical disorders as Alzheimer's disease and dementia (Bartus et al., 1982; Kasa et al., 1997). Habenular lesions, on the other hand, result in cognitive deficits, specifically, impairments in spatial memory, which is a behavior associated with schizophrenia (Lecourtier et al., 2004).

Dopamine modulates cholinergic activity throughout these systems via two general classes of DA receptors, the D1-class (D1 and D5 receptor subtypes) and the D2-class (D2, D3, and D4 receptor subtypes), which exert their biological effects via G-protein-coupled intracellular signaling pathways (Missale et al., 1998). The DA D1 receptor potentiates ACh release (Consolo et al., 1987; Damsma et al., 1990; Imperato et al., 1992; Acquas and Di Chiara, 1999) and a similar stimulatory effect on ACh release

has been reported for the DA D5 receptor (Hersi et al., 2000). In contrast, DA D2-class receptors attenuate ACh release (Stoof et al., 1987; Bertorelli and Consolo, 1990).

Northern blot analysis and in situ hybridization studies have reported DA D5 mRNA localization in limbic areas of the brain (Sunahara et al., 1991). Some of the highest levels of DA D5 mRNA (Yan and Surmeier, 1997) and receptor protein (Bergson et al., 1995) are found in striatal cholinergic interneurons along with very low levels of DA D1 mRNA (Le Moine et al., 1990; Yan and Surmeier, 1997; Nicola et al., 2000). Immunocytochemical localization of DA D5 receptors on striatal cholinergic neurons has been previously described (Rivera et al., 2002); however, DA D5 receptors have not been previously localized on any of the other cholinergic circuits investigated in the present study. Cholinergic interneurons also highly express the DA D2 receptor in the striatum (Alcantara et al., 2003). The high percentage of striatal cholinergic neurons reportedly expressing DA D5 and D2 receptors suggests that the majority of these neurons coexpress both receptor subtypes. Dopamine D1- and D2-class receptors can produce opposing, and occasionally synergistic effects at the cellular/molecular and behavioral levels (Stoof and Kebabian, 1981; Bertorello et al., 1990; Svenningsson et al., 2000; Fetsko et al., 2003). The synergistic effects associated with the colocalization of DA D1- and D2-class receptors in particular may contribute to the intracellular mechanisms that are critical for such phenomena as plasticity and learning (Kashihara et al., 1999; Silkis, 2001).

Given the evidence supporting the importance of DA D1-class receptor-mediated dopaminergic modulation of cholinergic neurotransmission (Acquas and Di Chiara, 2001), the present study investigated the neuroanatomical localization of the DA D5 receptor on cholinergic neurons of the rat forebrain and diencephalon. Dual labeling immunoperoxidase or immunofluorescence procedures, using antibodies directed against

choline acetyltransferase (ChAT) and the DA D5 receptor, were employed. Localization of the DA D5 receptor within these cholinergic networks provides evidence in support of a specific role for this receptor subtype in ACh release and, furthermore, will provide a better understanding of the neural mechanisms that underlie DA-ACh-mediated behaviors and related clinical disorders. (This study has been published: Berlanga, et al. 2005).

3.3 Methods

Tissue from eight adult male Sprague-Dawley rats (Simonsen Labs, Gilroy, CA) was used for dual immunoperoxidase or dual immunofluorescence procedures in this study. All efforts were made to minimize animal suffering and the number of animals used in the present study. Experimental procedures conformed to National Institutes of Health guidelines and were carried out under an institutionally reviewed and approved research protocol.

3.3.1 Animals

Eight Sprague-Dawley rats were used in this study. Six rats were used for the immunoperoxidase quantification procedures and two animals were used for the dual immunofluorescence procedures.

3.3.2 Tissue Preparation

Animals were administered an overdose of sodium pentobarbital (Nembutal; 100 mg/kg i.p.) and perfused transcardially with 60 ml of 0.1 M phosphate-buffered saline (PBS), pH 7.4, followed by 200 ml of 4% paraformaldehyde/0.1% glutaraldehyde in PBS, pH 7.4. The brains were removed and postfixed for 2 hours in 4% paraformaldehyde in PBS. Coronal sections were taken at a thickness of 100 μm on a vibratome, placed in 15% sucrose in PBS, and frozen in liquid nitrogen. Tissue sections

were then thawed and processed for light microscopy using single- or dual-labeling immunoperoxidase or immunofluorescence procedures for the DA D5 receptor and ChAT.

3.3.3 Dual Immunocytochemistry

Dopamine D5 receptor and ChAT dual immunoperoxidase labeling

Light microscopy dual labeling immunoperoxidase procedures were performed on free-floating, coronal tissue sections which were rinsed in 0.1 M PBS (2 × 10 minutes, 3 × 5 minutes) and preincubated for 1 hour in a PBS blocking solution containing 5% normal horse serum (NHS) and 0.01% hydrogen peroxide. Sections were then incubated simultaneously in a cocktail of both primary antibodies: affinity purified goat anti-dopamine D5 receptor polyclonal antibody (1:500; Chemicon, Temecula, CA) and affinity-purified mouse anti-ChAT monoclonal antibody (1:1,000; Chemicon) for 24 hours at 4°C. The DA D5 receptor polyclonal antibody is specific for the DA D5 receptor and exhibits no cross-reactivity with the DA D1 receptor or any other DA receptor types (Grandy et al., 1991; Sunahara et al., 1991; Tiberi et al., 1991). Sections were rinsed in PBS (3 × 5 minutes; and similarly rinsed after each step). The tissue was then incubated in secondary biotinylated donkey anti-mouse IgG antiserum (1:500; Jackson ImmunoResearch, West Grove, PA), diluted in PBS solution and 2% NHS for 2 hours. Subsequently, the tissue was incubated in an avidin-biotin peroxidase complex (ABC) (Vectastain Elite Kit; Vector, Burlingame, CA) for 1 hour. ChAT-immunoreactivity (IR) was visualized with 0.05% 3,3′-diaminobenzidine tetrahydrochloride (DAB) and 0.01% H_2O_2 in PBS, which resulted in a brown reaction product. The development time of DAB was minimal, so as not to obscure the second label. Sections were subsequently incubated in secondary biotinylated horse anti-goat IgG antiserum (1:200; Vector), diluted in PBS solution and 2% NHS for 1 hour, and then incubated in the ABC complex for 1 hour.

Dopamine D5 receptor-IR was visualized with the Vector SG substrate kit, resulting in a blue reaction product.

In order to verify the specificity of the ChAT/DAB and D5/SG dual labeling procedures, all combinations of single-labeled ChAT and DA D5 receptor visualized with either DAB or Vector SG were employed. Specific labeling for the respective antigens was observed in all of these procedures. Specifically, the two antibodies (ChAT and D5) were first processed as single labels to ensure that the individual labels were labeling specific cells in appropriate brain areas as previously reported in the literature. Once antibody specificity was verified, the antibodies were then used together to label DA D5 receptors on cholinergic neurons. Control sections for single- and dual-labeling procedures were processed identically with the exception that primary antibodies were omitted from the incubation solution. Single labeling was observed when one of the primary antibodies was omitted, and no staining was detected in the absence of both antibodies.

Two primary DA D5 receptor antibodies were used in this study for immunoperoxidase or immunofluorescence procedures. The DA D5 receptor antibody from Santa Cruz Biotechnology (Santa Cruz, CA) recognizes an 18-amino acid sequence (amino acids 455-472) on the carboxy terminal region of the fourth cytoplasmic loop (Ricci et al., 2001). This antibody recognizes a single band of appropriate molecular weight, 51 kDa, and shows the same general labeling pattern in tissue processed using the DA D5 receptor antibody from Chemicon, which recognizes a 19-amino acid sequence (amino acids 455-473) on the carboxy terminal region of the fourth cytoplasmic loop of the DA D5 receptor. All brain regions in this study were identified using The Rat Brain in Stereotaxic Coordinates by Paxinos and Watson (1998). The photomicrographs in each figure correspond to specific brain atlas illustrations included in Figures 3.1-3.6. All

photomicrograph images were processed for contrast and brightness with Adobe PhotoShop (San Jose, CA).

3.3.4 Dual Immunofluorescence

Dopamine D5 receptor and ChAT dual immunofluorescence labeling

Light microscopy dual labeling immunofluorescence procedures were performed on free-floating, coronal tissue sections. Sections were rinsed in 0.1 PBS (2 × 10 minutes, 3 × 5 minutes) and preincubated for 1 hour in a PBS blocking solution containing 5% normal donkey serum (NDS). Sections were then incubated simultaneously in a cocktail of both primary antibodies: affinity-purified goat anti-dopamine D5 receptor polyclonal antibody (1:50; Santa Cruz Biotechnology) and affinity-purified mouse anti-ChAT monoclonal antibody (1:1,000; Chemicon) for 48 hours at 4°C. Sections were rinsed in PBS (3 × 5 minutes). The tissue was then incubated in a cocktail of both secondary antibodies: Fluorescein (FITC) affinipure donkey anti-mouse IgG and Texas Red affinipure donkey anti-goat IgG (1:500; Jackson ImmunoResearch), diluted in PBS solution and 2% NDS for 1 hour. Sections were washed with PBS (3 × 5 minutes) and placed back in the primary cocktail for 1 hour followed by incubation in the secondary cocktail for 1 hour. The incubation in primary and secondary cocktails was repeated a third time with washes (PBS 3 × 5 minutes) between each hour incubation. The sections were then mounted and dried overnight and then coverslipped with Krystalon (EM Science Harleco, Gibbstown, NJ) mounting media. Control sections for dual labeling procedures were processed identically with the exception that primary antibodies were omitted from the incubation solution. Single labeling was observed when one of the primary antibodies was omitted, and no staining was detected in the absence of both antibodies.

Images were captured with a 40× objective (numerical aperture 1.25) using a Leica TCS 4D confocal laser-scanning microscope. FITC immunofluorescence was visualized with an excitation wavelength of 488 nm using an argon laser and Texas Red immunofluorescence was visualized with an excitation wavelength of 594 nm using an HeNe laser. Channels were acquired sequentially. FITC emission was collected between 500-570 nm. Texas Red emission was collected between 600-700 nm.

3.3.5 Quantitative Analysis

Quantitative analysis of cholinergic neurons was performed on dual ChAT/D5 immunoperoxidase-labeled tissue. Three digital images representing areas of 0.45 mm^2 were acquired from each of the brain areas of interest using a 10× objective on a Nikon Eclipse E800 light microscope. Each brain region was carefully identified based on reliable neuroanatomical landmarks using the atlas of Paxinos and Watson (Paxinos and Watson, 1998). Images were stored and analyzed. The percentage of cholinergic cells that expressed DA D5 IR was calculated for each sample area. An average number for the three sample areas per brain region was calculated for each animal. The group averages were subsequently calculated for each brain area of interest. The data are expressed as the mean and standard error of the mean (SEM) in Table 3.1.

3.4 Results

Dopamine D5 receptor labeling was detected on cholinergic neurons in each of the brain areas examined using dual labeling immunoperoxidase or immunofluorescence procedures. Both immunocytochemistry (ICC) procedures identified DA D5 labeling on cholinergic cell somata and dendrites. The immunoperoxidase procedure additionally provided the necessary cellular resolution to detect DA D5 labeling on axonal fibers. Quantitative analysis of ChAT/D5 coexpression reported in Table 3.1 was calculated

from immunoperoxidase-labeled tissue. The incidence of dual ChAT/D5 labeling and single ChAT or D5-labeled cells and fibers using the immunofluorescence procedure is reported for each brain area. Confocal laser immunofluorescence photomicrographs for five representative brain areas are presented. These areas include the primary motor cortex (M1) from the cerebral cortex, dorsal striatum (DS) from the neostriatum, horizontal diagonal band (HDB) and internal capsule/lateral globus pallidus (IC/LGP) from the basal forebrain, and rostral lateral hypothalamus (LH) from the diencephalon.

Table 3.1 . **Percentages of Cholinergic Neurons That Expressed the Dopamine D5 Receptor[1]**

Brain region	ChAT/D5 dual-labeled cells (%)
Cerebral cortex	
Infralimbic (IL)	68 ± 7
Prelimbic (PrL)	80 ± 4
Cingulate (Cg1)	59 ± 5
Motor	
Primary (M1)	60 ± 5
Secondary (M2)	61 ± 6
Somatosensory	
Primary (S1)	79 ± 6
Granular insular (GI)	73 ± 6
Agranular insular (AI)	75 ± 6
Neostriatum	
Dorsal striatum (DS)	93 ± 6
Nucleus accumbens (NAcc)	
Shell (Sh)	75 ± 10
Core (C)	85 ± 3
Islands of calleja (ICj)	79 ± 5
Olfactory tubercle (Olf)	75 ± 10
Basal forebrain	
Medial septal nucleus (MS)	81 ± 4
Vertical diagonal band (VDB)	72 ± 3
Horizontal diagonal band (HDB)	78 ± 3
Ventral pallidum (VP)	83 ± 5
Internal capsule/lateral globus pallidus (IC/LGP)	93 ± 3
Substantia innominata (SI)	88 ± 5
Magnocellular preoptic area (MCPO)	95 ± 3
Ansa lenticularis (AL)	71 ± 7
Diencephalon	
Medial habenula (MHB)	99 ± 0
Medial globus pallidus (MGP)	86 ± 11
Rostral lateral hypothalamus (LH)	82 ± 7

[1] The values listed reflect the group means and standard errors for the percent of cholinergic cells that expressed the DA D5 receptor for each brain region. ChAT = choline acetyltransferase.

Cholinergic interneurons of the cerebral cortex

Dopamine D5 receptor and ChAT dual immunoperoxidase labeling was examined in the infralimbic (IL), prelimbic (PrL), cingulate (Cg1), primary and secondary motor (M1 and M2), primary somatosensory (S1), granular insular (GI), and agranular insular (AI) cortices (Fig. 3.2A,B). Dopamine D5 receptor immunolabeling was present on the somata, dendrites, and axons of cortical cells within all of these cortical regions. Dopamine D5 receptor labeled fibers were observed in cortical layer I, the molecular layer of the cortex, which reportedly contains primarily cell processes and few cell bodies. Approximately 68% of the cholinergic cells in the IL cortex and 80% of the cholinergic cells in the PrL cortex revealed DA D5 receptor labeling. ChAT and DA D5 coexpression in the IL and PrL was observed primarily in layers II and III, with a few dual-labeled cells appearing occasionally in layers V and VI (Fig. 3.2C,E). Higher-magnification images of cells selected from the IL and PrL appear in Figure 3.2D,F, respectively. Quantification of dual-labeled cells in the Cg1 cortex revealed that 59% of the ChAT-positive cells were DA D5-positive. Dual-labeled cells were present in layers II-VI, with the highest density observed in layers II and III (Fig. 3.2G). A higher-magnification image of a dual-labeled cell from Cg1 is shown in Figure 3.2H. Dopamine D5-IR on ChAT-positive cells was evident in the primary and secondary motor cortices. Approximately 61% of the cholinergic cells in M2 and 60% in M1 revealed DA D5 receptor-IR (Fig. 3.2I-L). Dual immunofluorescence labeling procedures confirmed colocalization of the DA D5 receptor on cholinergic neurons and other cortical noncholinergic cells including large cells in layer V believed to be pyramidal cells in all of the cortical areas examined. Representative confocal laser photomicrographs from M1 cortex demonstrate coexpression of DA D5 and ChAT (Fig. 3.2M-O). Cholinergic cells with somatic and dendritic DA D5 receptor labeling were found throughout all layers of

S1 (Fig. 3.2P,Q). Quantification of dual-labeled cells in S1 revealed 79% of the ChAT cells in this cortical area were DA D5-positive. ChAT-positive cells were also found throughout all layers of GI and AI cortices, and these cells also exhibited DA D5 receptor labeling on their somata and dendrites (Fig. 3.2R-U). Approximately 73% of the ChAT-positive cells in the GI and 75% of the ChAT cells in the AI were DA D5-positive.

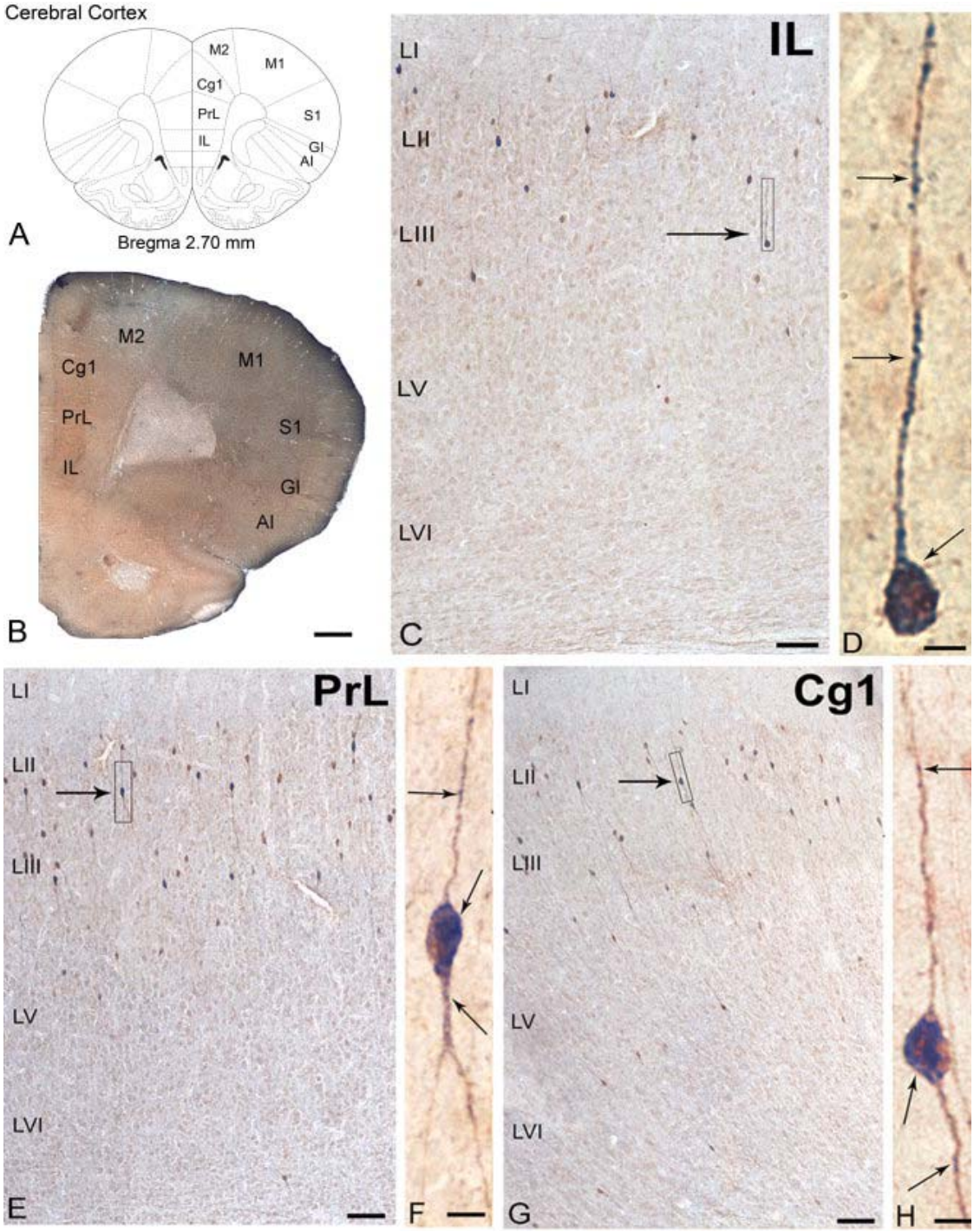

Figure 3.2. Dopamine D5 receptor localization on cholinergic neurons of the cerebral cortex.

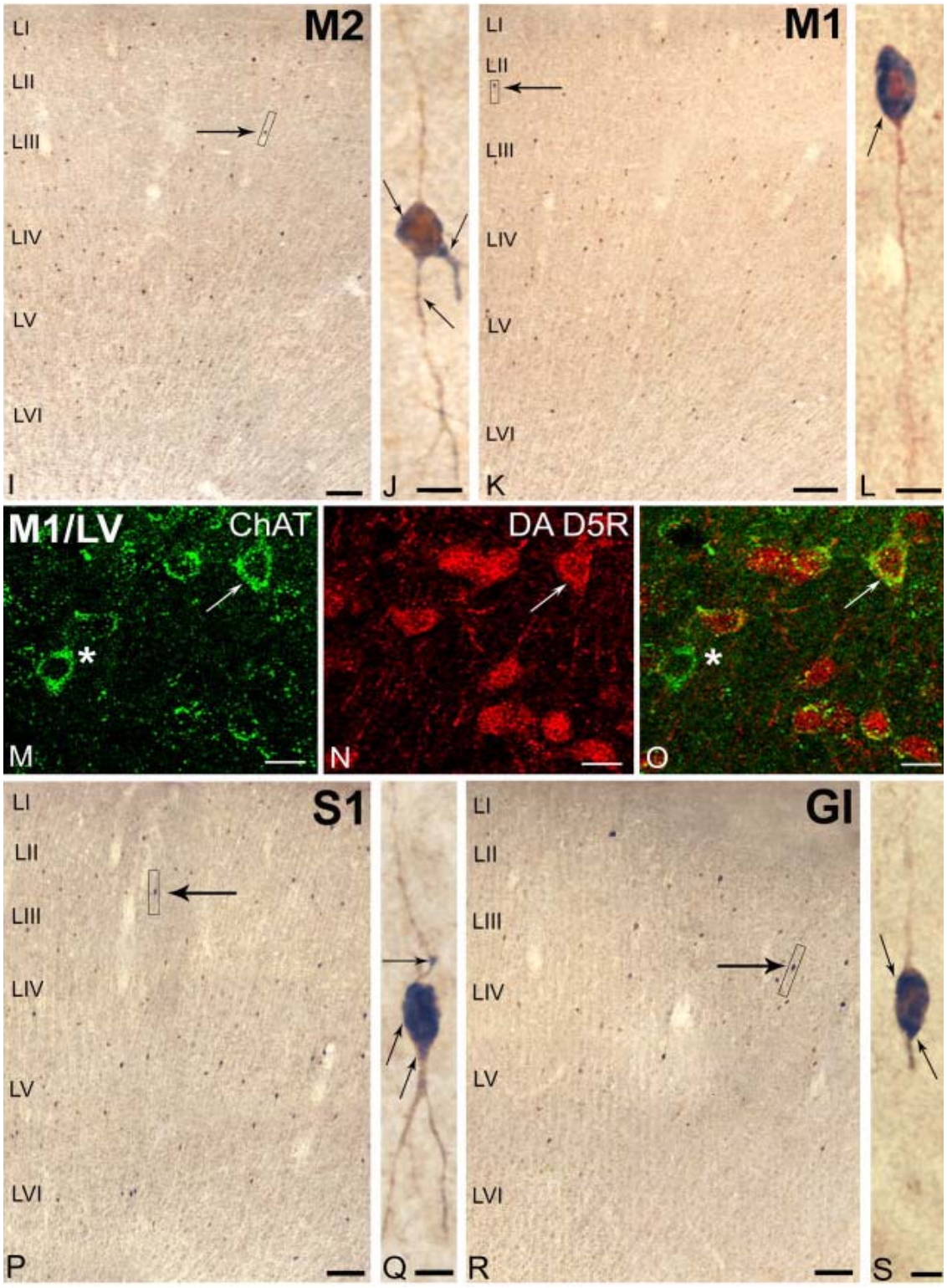

Figure 3.2 (continued).

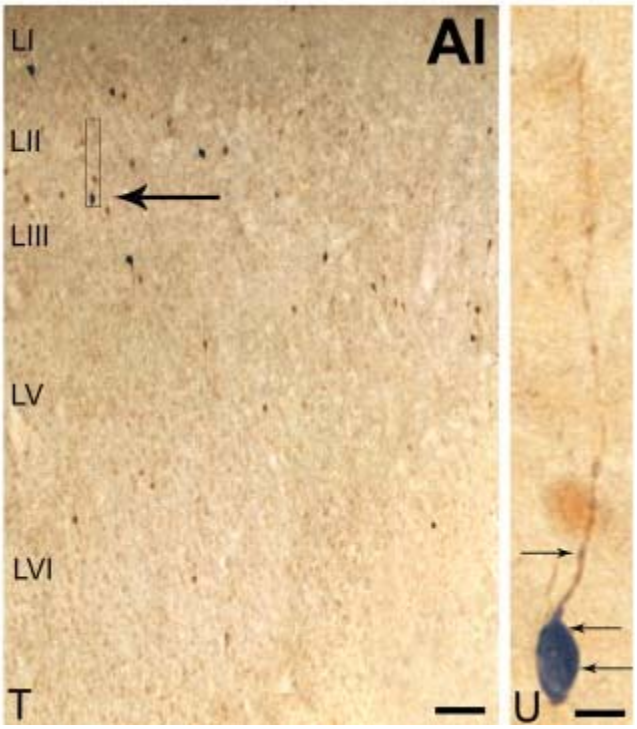

Figure 3.2 (continued). Photomicrographs of tissue from the cerebral cortex of the rat forebrain. A-H: Tissue was labeled for ChAT and DA D5 receptor using a dual labeling immunoperoxidase procedure. A: Schematic coronal section from the Paxinos and Watson atlas (Bregma 2.70 mm) identifies the cortical subregions of the rat forebrain where cholinergic neurons were examined. B: A low-magnification photomicrograph of a coronal section from the rat forebrain corresponding to the schematic in A. C: Dual immunoperoxidase labeled tissue revealed ChAT-IR cholinergic interneurons (brown) and DA D5 receptor labeling (blue) in the IL, PrL, and Cg1 cortices. Dopamine D5 receptor containing cholinergic neurons were observed primarily in layers II and III and occasionally in layers V and VI of the IL, PrL and Cg1. Representative dual-labeled cells from the IL, PrL, and Cg1 cortices (indicated by the boxes and black arrows) are shown at a higher magnification in D, F, and H. These cells reveal DA D5 receptor labeling (blue; black arrows) on the cell somata and process of the cholinergic neurons (brown) (D,F,H). IL, infralimbic cortex; PrL, prelimbic cortex; Cg1, cingulate cortex; M2, secondary motor cortex; M1, primary motor cortex; S1, primary somatosensory cortex; GI, granular insular cortex; AI, agranular insular cortex. I-S: Photomicrographs of tissue processed using dual labeling immunocytochemistry procedures revealed cholinergic interneurons (brown) and DA D5 receptor labeling (blue) in M2 (I), M1 (K), S1 (P), and GI (R) cortices. Dopamine D5 receptor containing cholinergic neurons were observed in cortical layers II through VI of M2 (I), M1 (K), S1 (P), and GI (R) cortices. Representative cells from M2, M1, S1, and GI (indicated by the boxes and black arrows) are shown at higher magnification (J,L,Q,S). Higher-magnification images of each cell

reveal DA D5 receptor labeling (blue; black arrows) on the cell bodies and processes of cholinergic neurons (brown) of M2 (J), M1 (L), S1 (P), and GI (R). High-magnification photomicrographs of dual immunofluorescence confocal laser photomicrographs from layer V of M1 reveal ChAT-IR neurons (FITC/green) (M) and DA D5 receptor labeled cells (Texas Red) (N). Coexpression of ChAT and D5 in these neurons is evident by the yellow label (O). White arrows indicate cells that revealed dual labeling and a white asterisk indicates a ChAT-IR cell that did not express the DA D5 receptor. M2, secondary motor cortex; M1, primary motor cortex; S1, primary somatosensory cortex; GI, granular insular cortex; ChAT, choline acetyltransferase; DA D5R, dopamine D5 receptor; LV, cortical layer five. T,U: Photomicrographs of tissue processed using dual labeling immunoperoxidase procedures revealed ChAT-IR neurons (brown) and DA D5 receptor labeling (blue) in the AI (T). Dopamine D5 receptor localization on cholinergic neurons was observed in layers II through VI of AI. A representative cell from the AI cortex (indicated by the box and black arrow) is shown at a higher magnification in U. A higher-magnification photomicrograph of the cell reveals DA D5 receptor labeling (blue; black arrows) on the cell body and process of the cholinergic neuron (brown) (U). AI, agranular insular cortex. Scale bars = 500 μm in B; 100 μm in C,E,G,T; 5 μm in D,F,H,J,L,Q,S,U; 200 μm in I,K,P,R; 15 μm in M-O.

Cholinergic interneurons of the striatum, nucleus accumbens, islands of Calleja, and olfactory tubercle

Cholinergic interneurons located in the dorsal striatum (DS) and nucleus accumbens (NAcc), including the shell (Sh) and core (C) compartments, as well as the neurons of the islands of Calleja (ICj) and the olfactory tubercle (Olf) (Fig. 3.3A,B) were found to express the DA D5 receptor on cell somata, dendrites, and axons. Dual-labeled cells were scattered throughout the DS and occasionally formed cell clusters. ChAT-IR axons in the striatum, distinguishable from dendrites based on a smaller diameter (axons measure less than 1 micron, whereas dendrites measure 1 micron or greater), prominent axonal varicosities, and a dense axonal arborization, revealed DA D5 labeling (Fig. 3.3C). The diameter of the axon illustrated in Figure 3.3C was estimated to be 0.6 μm, whereas dendrites found in the same area had diameters of ~1.5 μm. The presence of DA D5 receptor labeling was specifically localized on the varicosities of dual-labeled axons. The somata of striatal cholinergic interneurons stained heavily for the DA D5 receptor

and the dendrites of these cells revealed further labeling at circumscribed sites along the fibers. Quantification of dual-labeled cells revealed that 93% of ChAT labeled cells in the DS coexpressed the DA D5 receptor (Fig. 3.3D). Dual immunofluorescence labeling procedures confirmed localization of the DA D5 receptor on cholinergic neurons in the striatum, NAcc, ICj, and Olf. Representative confocal laser photomicrographs of the DS, demonstrate coexpression of DA D5 and ChAT (Fig. 3.3E,F). The immunofluorescence procedure also revealed DA D5 receptor labeling on other noncholinergic striatal cells, which presumably include medium spiny projection neurons (MSNs) and other interneurons. Similarly, single DA D5-labeled cells were also evident in the NAcc, ICj, and Olf. In the NAcc, 75% of the cholinergic neurons in the shell and 85% in the core were dual-labeled for ChAT and DA D5 (Fig. 3.3G,H). Cholinergic striatal interneurons in the DS were larger compared to cells in the shell and core compartments of the NAcc; however, cholinergic neurons frequently formed small clusters within the shell and core compartments. Approximately 79% of the cholinergic cells in the ICj and 75% in the Olf also showed dual labeling (Fig. 3.3I,J). Dual-labeled cells in the ICj were tightly clustered and typically smaller than striatal neurons.

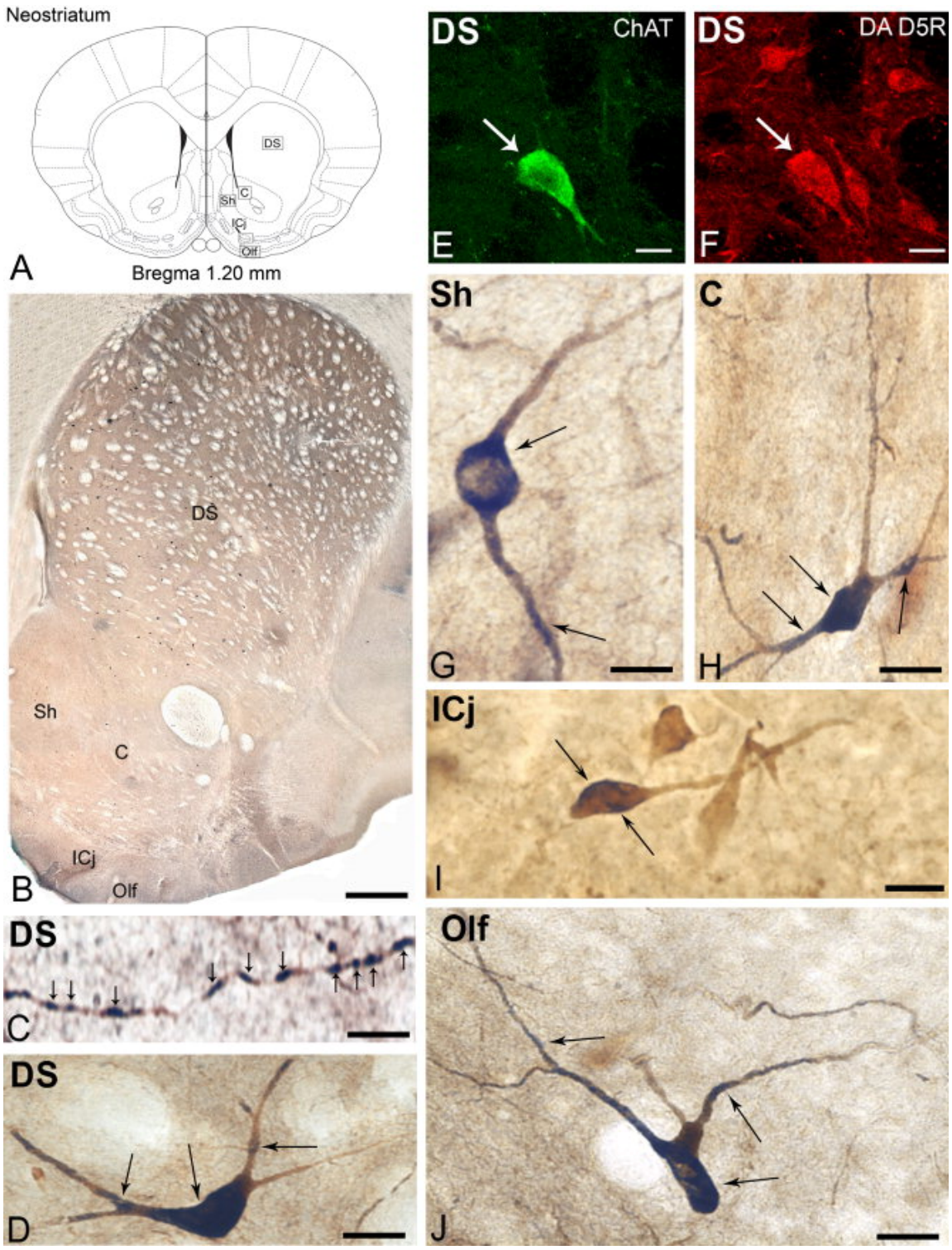

Figure 3.3. Dopamine D5 receptor localization on cholinergic neurons of the neostriatum. Photomicrographs from the neostriatum of the rat brain. Tissue was

labeled for ChAT and DA D5 receptor using dual labeling immunocytochemistry procedures. A: Coronal section from the Paxinos and Watson atlas indicating the areas where photomicrographs were taken of the neostriatum of the rat (Bregma 1.20 mm). B: A low-magnification photomicrograph of a ChAT/D5 immunolabeled coronal section corresponding to the schematic in A. C: A high-magnification photomicrograph of a dual-labeled axon from the dorsal striatum. Varicosities present on the neuronal process are typical characteristics of striatal axons. Arrows indicate DA D5 receptor labeling (blue) on the ChAT-IR cholinergic axon (brown). Receptor labeling was largely associated with the varicose regions of the axon. D: Photomicrograph of a cholinergic interneuron from the dorsal striatum. Dual immunolabeling revealed DA D5 receptor labeling (blue; black arrows) on cholinergic cells and on circumscribed regions of the cholinergic somata and dendritic processes. Confocal laser scanning photomicrographs show a neuron in the dorsal striatum that coexpressed ChAT (FITC/green) (E) and the DA D5 receptor (Texas Red) (F). White arrows indicate a dual-labeled ChAT/D5 cell. A few single-labeled D5-positive (noncholinergic) neurons are also visible in F. Dual ChAT/D5 immunolabeled cells were observed in the shell (G), and core compartments of the NAcc (H), island of Calleja (I), and olfactory tubercle (J). Black arrows indicate DA D5 receptor labeling (blue) on cholinergic cells (brown). DS, dorsal striatum; Sh, shell of the NAcc; C, core of the NAcc; ICj, island of Calleja; Olf, olfactory tubercle; ChAT, choline acetyltransferase; DA D5R, dopamine D5 receptor. Scale bars = 500 μm in B; 4 μm in C; 20 μm in D-J.

Cholinergic cells of the basal forebrain

The basal forebrain of the rat is made up of a collection of structures including the medial septal nucleus (MS), vertical diagonal band (VDB), horizontal diagonal band (HDB), ventral pallidum (VP), internal capsule (IC), lateral globus pallidus (LGP), substantia innominata (SI), magnocellular preoptic area (MCPO), and the ansa lenticularis (AL). Dopamine D5 receptor localization on cholinergic neurons was detected using dual labeling immunoperoxidase or immunofluorescence methods in each of these areas of the basal forebrain (Figs. 3.4-3.6).

Medial septal nucleus

The medial septal nucleus contained cells that were primarily round and small and were positioned in dense neuronal clusters (Fig. 3.4A,B). Dopamine D5 receptor labeling was evident on the somata and dendrites of cholinergic cells located within the MS (Fig.

3.4C). Quantification of dual-labeled cells revealed that 81% of the cholinergic cells in the MS expressed the DA D5 receptor. ChAT-IR axonal fibers exhibiting DA D5 receptor labeling were also noted in this area. Many single DA D5-labeled fibers and cells were observed in this region.

Vertical and horizontal limbs of the diagonal band

Cholinergic projection cells were positioned in dense neuronal clusters (Fig. 3.4B) in the limbs of the VDB (Fig. 3.4D) and HDB (Fig. 3.4E). The cells appeared to be small and the somata were generally round, with extensive visible fibers, similar to the cholinergic cells of the MS. Approximately 72% of cholinergic cells in the VDB and 78% in the HDB revealed dense DA D5 receptor labeling. Dual-labeled varicose axonal fibers were also evident in this area. Dual labeling immunofluorescence procedures confirmed the presence of the DA D5 receptors on cholinergic neurons in all areas of the basal forebrain. Representative confocal laser photomicrographs of the HDB demonstrate coexpression of DA D5 and ChAT (Fig. 3.4F,G). The immunofluorescence procedure also revealed DA D5 receptor labeling on other noncholinergic cells in the HDB.

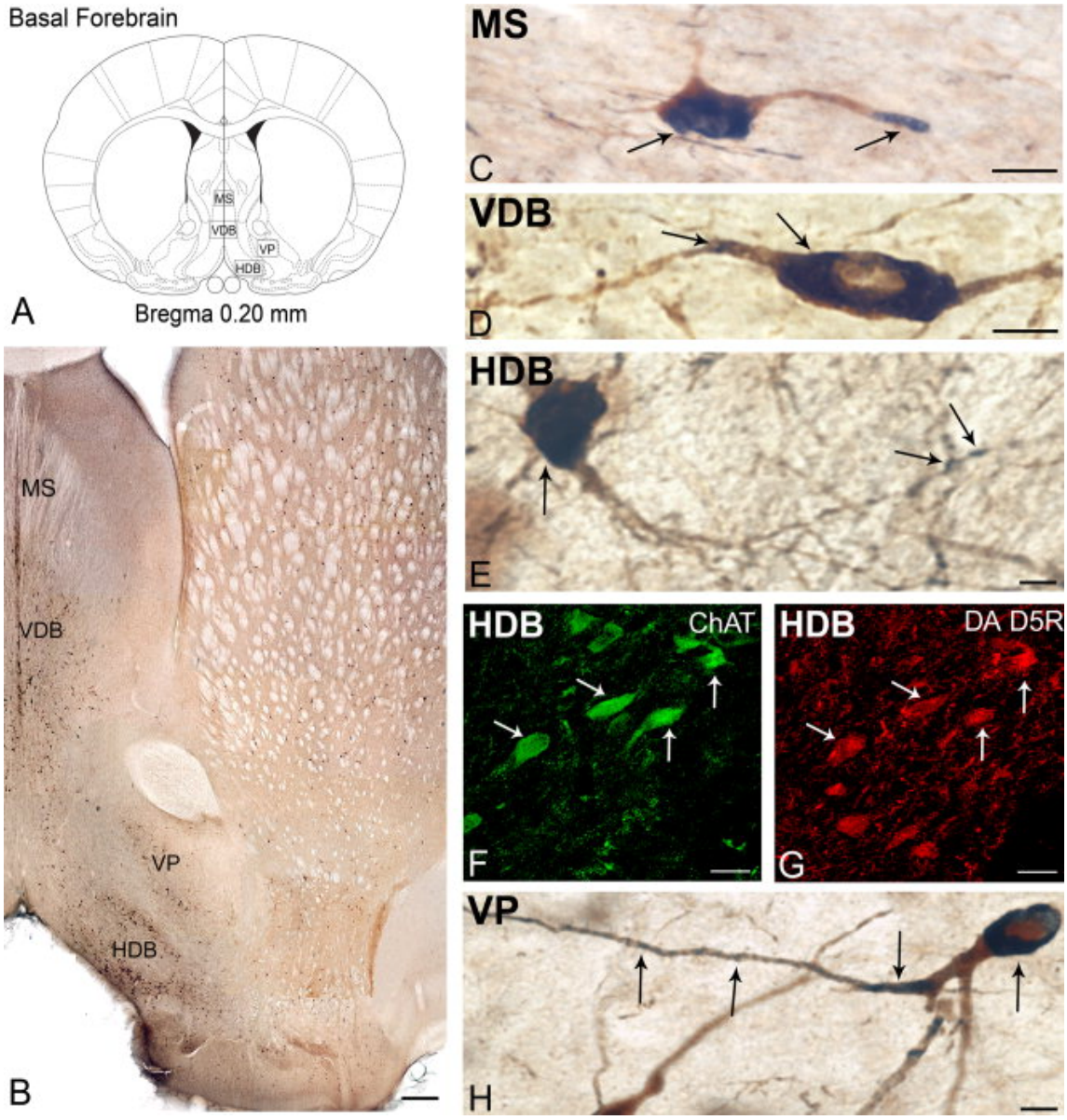

Figure 3.4. Dopamine D5 receptor localization on cholinergic neurons of the rostral basal forebrain. Photomicrographs from the basal forebrain. Tissue was labeled for ChAT and DA D5 receptor using dual labeling immunocytochemistry procedures. A: Coronal section from the Paxinos and Watson atlas indicating the areas where photomicrographs were taken from the basal forebrain (Bregma 0.20 mm). B: A low-magnification photomicrograph taken of a coronal section corresponding to the schematic in A. Dual ChAT/D5 immunolabeled cells were observed in the medial septal nucleus (C), vertical limb of the diagonal band (D), and horizontal limb of the diagonal band (E). Black arrows indicate DA D5 receptor labeling (blue) on cholinergic cells (brown). F,G:

A pair of dual immunofluorescence confocal laser photomicrographs from the horizontal limb of the diagonal band revealed cells that coexpressed ChAT (FITC/green) (F) and the DA D5 receptor (Texas Red) (G). White arrows indicate the dual-labeled neurons. A few single-labeled ChAT-IR neurons are visible in F and a few single-labeled D5-positive neurons and fibers are visible in G. Photomicrograph of a cholinergic cell from the ventral pallidum (H) also revealed DA D5 labeling (blue; black arrows) on cholinergic neurons (brown). MS, medial septal nucleus; VDB, vertical diagonal band; HDB, horizontal diagonal band; VP, ventral pallidum; ChAT, choline acetyltransferase; DA D5R, dopamine D5 receptor. Scale bars = 500 μm in B; 10 μm in C-E,H; 20 μm in F,G.

Ventral pallidum

The morphology and size of VP cholinergic cells were similar to those in the diagonal band and the MS. These cells were typically positioned in clusters; however, they were not situated in dense clusters as those observed in the MS and diagonal band. Dopamine D5 receptor labeling was visible on the somata and dendrites of 83% of these cells (Fig. 3.4H). Dual-labeled axons were also noted in this area. In addition, many DA D5 single-labeled fibers and cells were also observed in this region.

Internal capsule and lateral globus pallidus

The IC and the LGP contained clusters of cholinergic cells (Fig. 3.5A,B). The cells were observed to border the edges of the IC and the LGP. Dopamine D5 receptor localization was evident on the somata, dendrites, and axons of 93% of ChAT-IR cells of this region (Fig. 3.5C). Dual labeling immunofluorescence procedures confirmed colocalization of the DA D5 receptor on cholinergic neurons in this area. Representative confocal laser photomicrographs of the IC/LGP demonstrate coexpression of DA D5 and ChAT (Fig. 3.5D,E). No single DA D5-labeled cells were observed in this region.

Substantia innominata

Cells in the SI (Fig. 3.5F) were similar in size and morphology to cholinergic neurons of the VP. Dopamine D5 receptor labeling was observed on the somata,

dendrites, and axons of 88% of the cholinergic neurons. Dopamine D5 receptor labeling was also observed on other noncholinergic cells and fibers in this region.

Magnocellular preoptic area

Cells in the MCPO (Fig. 3.5G) were similar in morphology and size to those cells observed in the VP and SI. Approximately 95% of cholinergic cells in the MCPO revealed DA D5 receptor labeling. Dopamine D5 IR was found on the cell bodies and dendrites of those cells. Dopamine D5 receptor labeling was also observed on other noncholinergic cells and fibers in this region.

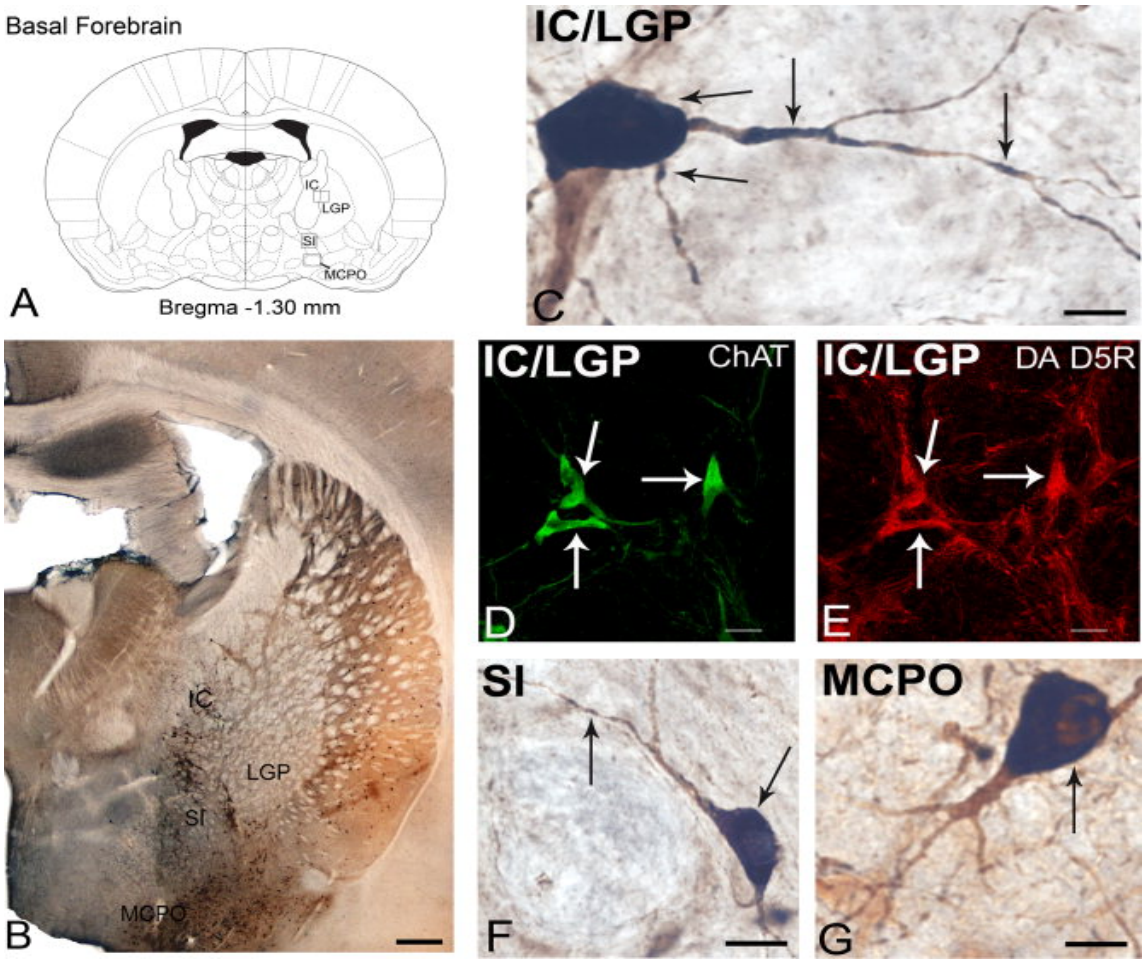

Figure 3.5. Dopamine D5 receptor localization on cholinergic neurons of the basal forebrain. Photomicrographs of tissue from the basal forebrain. Tissue was labeled for ChAT and DA D5 receptor using dual labeling immunocytochemistry procedures. A: Coronal section from the Paxinos and Watson atlas indicating the areas where photomicrographs were taken from the basal forebrain (Bregma -1.30 mm). B: A low-magnification photomicrograph of a dual ChAT/D5-immunolabeled coronal section corresponding to the schematic in A. C: A dual-labeled cholinergic neuron located in the border between the internal capsule and lateral globus pallidus. A pair of confocal laser scanning photomicrographs show dual-labeled neurons located at the border of the IC and LGP that were IR for ChAT (FITC/green) (D) and the DA D5 receptor (Texas Red) (E). White arrows indicate coexpression of ChAT and D5 in these neurons. Photomicrograph of a cholinergic cell from the substantia innominata (F) and the magnocellular preoptic area (G) also revealed DA D5 labeling (blue; black arrows) on cholinergic neurons (brown). IC, internal capsule; LGP, lateral globus pallidus; SI, substantia innominata; MCPO, magnocellular preoptic area; ChAT, choline acetyltransferase; DA D5R,

dopamine D5 receptor. Scale bars = 500 μm in B; 15 μm in C,F; 20 μm in D,E; 10 μm in G.

Ansa lenticularis

The AL, which is adjacent to the nigrostriatal bundle, the medial forebrain bundle, and the lateral hypothalamus, also contained clusters of cholinergic cells (Fig. 3.6A-C). These cells were smaller than cells in the MCPO and were tightly packed together. Approximately 71% of the cells in this region coexpressed ChAT and DA D5 receptors. These cells exhibited DA D5 receptor labeling on their somata and dendrites. Dual-labeled axons were also observed in this region. No single DA D5-labeled cells were observed in this region.

Cholinergic cells of the diencephalon

The cholinergic cells of the diencephalon were spread across three different regions, including the medial habenula (MHB), medial globus pallidus (MGP), and rostral lateral hypothalamus (LH). Dopamine D5 receptor localization on cholinergic neurons was observed in each of these areas using dual labeling immunoperoxidase or immunofluorescence procedures (Fig. 3.6).

Medial habenula

The greatest cholinergic cell density was observed in the MHB (Fig. 3.6D). Cells were small and round and tightly packed together, with few visible fibers. Approximately 99% of these cells stained for the DA D5 receptor. Labeling on the fibers of these cells was difficult to discern because the cells were so tightly packed together. No single DA D5-labeled cells were observed in this region.

Medial globus pallidus

Cholinergic cells of the MGP (Fig. 3.6E) were similar in size and morphology to cells located along the edge of the LGP. Cholinergic cells in the MGP, however, were

more evenly distributed in this brain area as compared to the LGP. Dopamine D5 receptor labeling was observed on the somata, dendrites, and axons of 86% of these cholinergic cells as well as on many noncholinergic cells.

Rostral lateral hypothalamus

The cholinergic cells of the rostral LH (Fig. 3.6F) were morphologically similar and in close proximity to the cholinergic cells of the MGP. Dopamine D5 receptor labeling was evident on the somata, dendrites, and axons of 82% of ChAT-IR neurons in this region. Dopamine D5 receptors also appeared on noncholinergic neurons in this brain area (Fig. 3.6G,H). Dual labeling immunofluorescence procedures confirmed colocalization of the DA D5 receptor on cholinergic neurons in this area. Representative confocal laser photomicrographs of the LH demonstrate coexpression of DA D5 and ChAT (Fig. 3.6G,H). The immunofluorescence procedure also detected DA D5 receptor labeling on other noncholinergic cells in the LH.

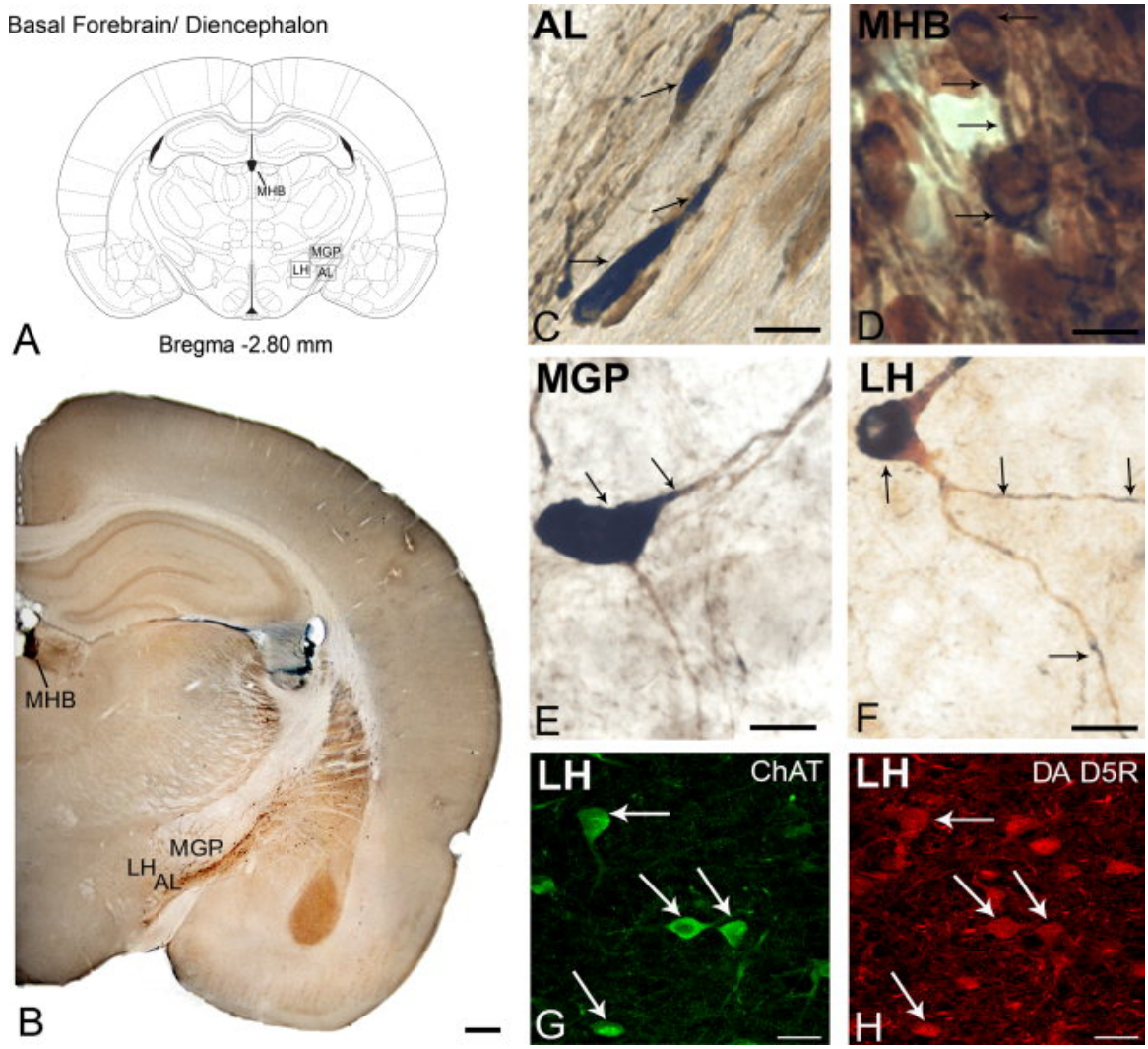

Figure 3.6. Dopamine D5 receptor localization on cholinergic neurons of the basal forebrain and diencephalon. Photomicrographs of tissue from the basal forebrain and diencephalon. Tissue was labeled for ChAT and DA D5 receptor using dual labeling immunocytochemistry procedures. A: Coronal section from the Paxinos and Watson atlas indicating the areas where photomicrographs were taken from the basal forebrain and diencephalon (Bregma -2.80 mm). B: A low-magnification photomicrograph of a coronal section corresponding to the schematic in A. Dual-labeled ChAT/D5-positive cells were observed in the ansa lenticularis (C), medial habenula (D), medial globus pallidus (E), and the lateral hypothalamus (F). Dopamine D5 receptors (blue; black arrows) labeled the somata of cholinergic neurons (brown) in these brain regions. Dopamine D5 receptor

labeling was also observed on dendrites of these cells and on axons located within these areas. A pair of confocal laser scanning photomicrographs show neurons located in the lateral hypothalamus that were dual-labeled for ChAT (FITC/green) (G) and the DA D5 receptor (Texas Red) (H). White arrows indicate D5-positive cholinergic cells. Single-labeled DA D5 receptor cells and fibers are also visible in H. AL, ansa lenticularis; MHB, medial habenula; MGP, medial globus pallidus; LH, lateral hypothalamus. Scale bars = 500 μm in B; 10 μm in C; 5 μm in D; 15 μm in E,F; 20 μm in G,H.

3.5 Discussion

The cholinergic systems of the cerebral cortex, striatum, basal forebrain, and diencephalon have widespread connections, affecting diverse target structures and a myriad of cognitive-, limbic-, and motor-related functions. Results from this study revealed that the majority of cholinergic cells in each of these areas express the DA D5 receptor. More specifically, this study reports DA D5 receptor localization on somata, dendrites, and axons of cholinergic cells in each of the brain areas examined.

Dual labeling immunoperoxidase or immunofluorescence procedures were employed in this study to visualize DA D5 receptors on cholinergic cells in the forebrain and diencephalon. Tissue from the brainstem and mesencephalon was not included in this study, but, given the importance of these cell groups, will be included and examined in future studies. Both techniques revealed patterns of labeling that were consistent with those reported in the literature for DA D5 IR, ChAT-IR, or coexpression of both in the rat brain. For example, striatal cholinergic interneurons, which are known to express among the highest DA D5 receptor mRNA (Yan et al., 1997; Yan and Surmeier, 1997) and receptor protein levels (Bergson et al., 1995; Rivera et al., 2002) throughout the striatum (Nicola et al., 2000) showed a high incidence of DA D5 receptor labeling in the present study using either ICC procedure. Noncholinergic striatal cells including MSNs and other interneurons that have been reported to express low levels of DA D5 mRNA or D5 receptor protein (Nicola et al., 2000; Rivera et al., 2002) were detected only by the

immunofluorescence procedure. The immunofluorescence procedure detected DA D5 IR in noncholinergic cells in most of the brain areas examined with the exception of the IC/LGP, AL, and MHB, which did not reveal single DA D5-immunolabeled cells. Quantification of the percent of cholinergic neurons that expressed DA D5 labeling (presented in Table 3.1) was calculated using the dual labeling immunoperoxidase procedure. These values, however, may be considered conservative since this procedure appeared to detect DA D5-positive cells only in instances where the cells contained sufficiently high levels of the receptor. Among the areas that showed the lowest values of DA D5 receptor and ChAT coexpression, using this immunoperoxidase procedure, included the cingulate and motor cortices, whereas the highest incidence of DA D5-positive cholinergic cells was evident in the DS, IC/LPG, MCPO, and MHB.

The circumscribed localization of DA D5 receptors on cholinergic cells and fibers may provide evidence of receptor trafficking and clustering at specific sites along cell somata and dendritic and axonal processes of these cells. Evidence of somatic DA D5 receptor clustering in our study is consistent with reported DA D5 clustering in primate cortical pyramidal cells (Paspalas and Goldman-Rakic, 2004). Somatic DA D5 receptor immunolabeling may be attributed to the fact that the primary site of protein synthesis is believed to take place in the soma, although mounting evidence suggests that translation may also occur in axons (Piper and Holt, 2004). Additionally, electron microscopy (EM) work by Dimova et al. (1993) reported that striatal cholinergic neurons receive occasional synaptic contact from tyrosine hydroxylase (TH)-positive axon terminals on their somata and dendrites, with the majority of synapses occurring on proximal cholinergic dendrites. Thus, DA D5 receptors may be mediating ACh neurotransmission by clustering at DA synaptic sites along the soma and dendritic processes. Pickel and Chan (1990), however, reported that only about 1% of TH-positive axons in the striatum and nucleus accumbens

are apposed to, or form synapses with, the perikarya or dendrites of ChAT-labeled cells. Thus, the major influence on DA receptors located on these cholinergic neurons may be nonsynaptically at extrasynaptic sites via volume neurotransmission. Bergson et al. (1995) further substantiate this notion through EM evidence of DA D5 receptor localization at extrasynaptic sites (Sesack et al., 2003).

In the present study, some of the cholinergic axons showed DA D5 receptor labeling, particularly on axonal varicosities. Cholinergic axons can be identified at the light microscopic level based on their small diameter (measuring less than 1 micron), as compared to dendrites (measuring 1 micron or greater) (Wainer et al., 1984; Phelps et al., 1985; Contant et al., 1996), as well as their distinct branching pattern, typically a plexus of axonal fibers (Bennett and Wilson, 1999). Further examination of axonal varicosities at the EM level revealed the presence of aggregated synaptic vesicles and stereological quantification of the volume of varicosities receiving synaptic contact revealed a synaptic incidence of 8.8% (Contant et al., 1996). Low levels of synaptic incidence at axonal varicosities suggest that ACh release may occur either at synaptic or asynaptic sites. The positioning of DA D5 receptors at these varicosities further suggests DA modulation of ACh release from these sites. A study combining VAChT (a marker that labels cholinergic axon terminals more intensely than ChAT) and the DA D5 receptor would provide a more thorough analysis of DA D5 receptor localization on cholinergic axons. Studies examining the precise functional role of these receptors on ACh release, however, have yet to be conducted.

The DA D5 receptor has been investigated in a number of cognitive- and motor-related disorders, including schizophrenia, Tourette's syndrome, antisocial personality disorder, attention-deficit hyperactivity disorder, and drug abuse (Barr et al., 1997; Vanyukov et al., 1998; Barr et al., 2000; Filip et al., 2000; Vanyukov et al., 2000; Muir et

al., 2001). The specific role of the DA D5 receptor, however, is not yet clear. One of the limitations of research focused on uncovering the precise function of the DA D5 receptor is the lack of specific DA D5 receptor subtype agonists or antagonists. Therefore, there is no direct evidence correlating the DA D5 receptor with a specific cellular function or behavioral effect. One means of inferring the role of DA D5 has been to investigate the role of D1-class agonists on striatal cholinergic neurons because of the preponderant localization of DA D5 (and not D1) on these neurons (Suzuki et al., 2001). Knockout animals have also recently been used to study the effects of DA D5 receptors (Centonze et al., 2003; Elliot et al., 2003); however, in combination with those studies, specific agonists or antagonists are required to more precisely investigate the role of the DA D5 receptor subtype.

Dopamine D1 and D5 receptors are receptor homologs, sharing the highest degree of homology within their transmembrane segments. However, these two receptor subtypes possess some structural and functional differences. For example, these two receptor subtypes differ by two amino acids in the C-terminal region of the third cytoplasmic loop (Charpentier et al., 1996). This structural difference is believed to be responsible for the differences in binding affinity observed between these two receptor subtypes (Sugamori et al., 1998). The DA D5 receptor has a binding affinity to DA that is 10 times greater than the DA D1 receptor, as well as a more potent intracellular G-protein-coupling mechanism (Grandy et al., 1991; Sunahara et al., 1991; Kimura et al., 1995). Therefore, while the DA D5 receptor is classified functionally as a D1-class receptor, this receptor subtype possesses some structural and functional differences requiring further investigation.

The cellular transduction mechanisms triggered by the activation of DA D1 and D5 receptors, in turn, will contribute to a wide range of cellular and behavioral outcomes.

For example, it has recently been reported that the DA interacting protein calcyon facilitates DA D1 receptor release of intracellular Ca2+ through the involvement of an inositol 1,4,5-triphosphate-gated (InsP3) cascade (Bergson et al., 2003). Intracellular Ca2+ release in turn has been linked to plasticity and learning, more specifically LTP (Yeckel et al., 1999), and spine plasticity (Korkotian and Segal, 1999). It is further posited that the DA D5 receptor may be similarly involved given the 80% sequence similarity that it shares with the DA D1 receptor calcyon-binding motif. In general, an understanding of such DA signaling molecular mechanisms will have important clinical implications. For example, an increase in calcyon (100%) has been observed in the prefrontal cortex of schizophrenic patients (Koh et al., 2003). Additionally, Paspalas and Goldman-Rakic (2004) have recently provided ultrastructural evidence that DA D5 (but not D1 or D2) receptors are localized in the perisomatic plasma membrane of prefrontal cortical cells which form extrasynaptic microdomains with InsP3-gated calcium stores of subsurface cisterns and mitochondria. Such discrete receptor positioning may serve to translate extracellular DA signals into receptor-specific spatiotemporal downstream signaling similar to the "one-to-one" spatiotemporal properties attained through chemical synapses.

The present study provides supporting evidence that the DA D5 receptor subtype may serve as an important neuroanatomical substrate involved in mediating DA influences on ACh neurotransmission throughout the brain. Findings from this study in addition to future investigations of the functional role of the DA D5 receptor may reveal an important neural substrate for dopaminergic signaling that has been largely overlooked. In particular, it will become important to gain an understanding of the positioning of these receptors at specific sites on cholinergic neurons and other cell types and their relationship to synaptic and volume transmission influences. The cellular

positioning together with the high affinity properties of DA D5 receptors will define the precise circumstances in which these receptors are activated. The high affinity properties of the DA D5 receptor, for example, would favor DA signaling at low neurotransmitter levels and potentially long-range spillover or volume transmission influences. Furthermore, this receptor would be differentially responsive to changes in DA levels that occur as a result of behaviorally guided tonic versus phasic firing of mesencephalic DA neurons, as described for such a behavioral phenomenon as learning and such disorders as drug abuse and schizophrenia (Grace, 1991; Schultz, 2001; Phillips et al., 2003).

In conclusion, an understanding of the localization of DA D5 receptors at defined sites on cholinergic cells and their relationship to functionally defined cellular microcircuits and intracellular transduction pathways will provide a greater understanding of the precise role that DA D5 receptors play in a variety of cognitive-, limbic-, and motor-related behaviors. These findings will also serve to guide the development of improved site-specific targeted pharmacotherapeutic strategies and cognitive-behavioral treatments for a variety of neuropsychiatric disorders.

Chapter 4: Localization of Dopamine D2 Receptors on Cholinergic Interneurons of the Dorsal Striatum and Nucleus Accumbens of the Rat

4.1 Abstract

Striatal cholinergic interneurons located in the dorsal striatum and nucleus accumbens are amenable to influences of the dopaminergic mesolimbic pathway, which is a pathway involved in reward and reinforcement and targeted by several drugs of abuse. Dopamine and acetylcholine neurotransmission and their interactions are essential to striatal function, and disruptions to these systems lead to a variety of clinical disorders. Dopamine regulates acetylcholine release through dopamine receptors that are localized directly on striatal cholinergic interneurons. The dopamine D2 receptor, which attenuates acetylcholine release, has been implicated in drug relapse and is targeted by therapeutic drugs that are used to treat a variety of neurological disorders including Tourette's syndrome, Parkinson's disease and schizophrenia. The present study tested the hypothesis that cholinergic interneurons in the dorsal striatum and nucleus accumbens express the dopamine D2 receptor. This study provides the first direct evidence for the localization of dopamine D2 receptors on striatal cholinergic interneurons of the rat brain using dual labeling immunocytochemistry procedures. Using light microscopy, dopamine D2 receptors were localized on the cell somata and dendritic and axonal processes of striatal cholinergic interneurons in the dorsal striatum and nucleus accumbens of the rat brain. These findings provide a foundation for understanding the specific roles that cholinergic neuronal network systems and interacting dopaminergic signaling pathways play in striatal function and in a variety of clinical disorders including drug abuse and addiction.

4.2 Introduction

This study focused on dopamine–acetylcholine interactions in the dorsal striatum and the nucleus accumbens of the rat brain. The dorsal striatum which integrates a variety of cortical and limbic information is involved primarily in the regulation of motor function. The nucleus accumbens (NAcc), which is a major area of the ventral striatum, has been an area of intensive investigation as central to the rewarding and locomotor effects of drugs of abuse and other naturally rewarding behaviors. The dopaminergic mesolimbic pathway, which originates in the ventral tegmental area (VTA) and provides extensive afferent projections to the dorsal striatum and NAcc, is targeted by several drugs of abuse including cocaine, amphetamine, heroin, morphine, nicotine and alcohol (Self and Nestler, 1995; Berke and Hyman, 2000). These dopaminergic projections maintain distinct topography within the subdivisions of the dorsal striatum, and the two major divisions of the NAcc, the shell and core (Gerfen et al., 1987; Heimer et al., 1991; Usuda et al., 1998). These subdivisions, therefore, have distinct structure, connectivity, and morphology and thus subserve separate reward and motor-related phenomena.

This study focused on cholinergic interneurons of the dorsal striatum and NAcc, which are amenable to dopaminergic mesolimbic influences through the activation of dopamine (DA) receptors that are located directly on these neurons. Cholinergic interneurons are important neuronal integrators and modulators of striatal function and dysfunction. These neurons possess key receptors that are linked to molecular signaling pathways critical for plasticity (Calabresi et al., 2000). Striatal cholinergic interneurons also express long-term potentiation (LTP) (Suzuki et al., 2001) and play a unique role in reward mediated associative learning (Aosaki et al., 1994). Furthermore, these local circuit neurons have been demonstrated to exert powerful influences on medium spiny output neurons (MSNs) and thus on overall striatal signaling (Howe and Surmeier, 1995).

More specifically, cholinergic interneurons have been reported to modulate corticostriatal NMDA receptor signaling onto MSNs and the LTP that is expressed by these striatal output neurons (Calabresi et al., 2000; Alcantara et al., 2001).

The effects of DA actions on cholinergic interneurons are mediated through two types of G-protein coupled receptors: D1-class (D1, D5) and D2-class (D2, D3, D4). The cellular processes associated with these receptor subtypes have been linked directly to neural plasticity and learning as well as drug-seeking behavior (Self et al., 1996; Watanabe and Kimura, 1998; Nicola et al., 2000; De Vries and Shippenberg, 2002). It has been shown that dopamine exerts mainly opponent action on D1- and D2-like dopamine receptors. Acetylcholine (ACh) release is increased by dopamine D1-like agonists (Consolo et al., 1999), while dopamine D2 receptor activation has been shown in both in vivo and in vitro studies to inhibit ACh release (Lehmann and Langer, 1983; Stoof et al., 1987; Bertorelli and Consolo, 1990; Damsma et al., 1990; Drukarch et al., 1990). In addition to their opposing effects, however, synergistic effects can also occur through the co-activation of dopamine D1 and D2 receptors (LaHoste and Marshall, 1993; Hu and White, 1994; Kashihara et al., 1999).

The aim of the present study was to identify the distribution of dopamine D2 receptors on cholinergic interneurons of the dorsal striatum and NAcc in the rat brain, using light microscopy with immunocytochemical dual labeling procedures. This study provides evidence of a neuroanatomical substrate for possible reward and motor mediated DA influences onto dorsal striatal and accumbal cholinergic neuronal network systems. The findings from this work should lead to a better understanding of cholinergic circuitry, specifically that involving DA signaling onto cholinergic neurons and the effects of acetylcholine neurotransmission on overall striatal function and dysfunction. (This study has been published: Alcantara et al., 2003).

4.3 Methods

Experimental procedures conformed to National Institute of Health guidelines and were carried out under an institutionally reviewed and approved research protocol.

4.3.1 Animals

Twelve adult male Sprague–Dawley rats (Simonsen Labs) were used in this study.

4.3.2 Tissue Preparation

Animals were administered an overdose of sodium pentobarbital (Nembutal; 100 mg/kg i.p.) and perfused transcardially with 60 ml of 0.1 M phosphate buffered saline (PBS), pH 7.4 followed by 200 ml of 4% paraformaldehyde/0.1% glutaraldehyde in PBS, pH 7.4. The brains were removed and post-fixed for 1 h in 4% paraformaldehyde in PBS. Vibratome sections were taken at a thickness of 70 μm, placed in 15% sucrose in PBS, and frozen in liquid nitrogen. Tissue sections were then thawed and processed for light microscopy utilizing single or dual labeling immunocytochemistry (ICC) procedures for dopamine D2 receptor and choline acetyltransferase (ChAT).

4.3.3 Light Microscopy Immunocytochemistry

D2/Vector SG and ChAT/DAB immunolabeling

Light microscopy dual labeling immunocytochemical procedures were performed on free-floating, coronal tissue sections which were rinsed in 0.1 M PBS (3×10 min) and preincubated for 1 h in a PBS blocking solution containing 5% normal goat serum (NGS). Sections were then incubated simultaneously in a cocktail of both primary antibodies: rabbit dopamine D2 receptor polyclonal antibody (1:350; Chemicon) and mouse ChAT monoclonal antibody (1:1000; Chemicon) for 24 h at 4 °C. The preparation, characterization, and specificity of the dopamine D2 receptor antibody have been previously described (Bunzow et al., 1988; Farooqui et al., 1991; Brock et al., 1992;

Sakata et al., 1992). Sections were rinsed in PBS (4×5 min; and similarly rinsed after each step unless otherwise specified). The tissue was then incubated in secondary biotinylated donkey anti-mouse IgG antiserum (1:500; Jackson ImmunoResearch), diluted in 2% NGS-PBS for 2 h, and then incubated in an avidin–biotin peroxidase complex (ABC Vectastain Elite Kit; Vector) for 1 hr. ChAT-immunoreactivity (IR) was visualized with 0.05% 3,3′-diaminobenzidine tetrahydrochloride (DAB) and 0.01% H_2O_2 in PBS which resulted in a brown reaction product. Sections were subsequently incubated in secondary biotinylated goat anti-rabbit IgG antiserum (1:200; Vector) diluted in 2% NGS-PBS for 1 h and then incubated in the ABC complex for 1 hr. Dopamine D2 receptor immunoreactivity was visualized with the Vector SG substrate kit, resulting in a blue reaction product.

In order to verify the specificity of the ChAT/DAB and D2/SG dual labeling procedures, all combinations of single labeled ChAT and dopamine D2 receptor visualized with either DAB or Vector SG were employed. Specific labeling for the respective antigens was observed in all of these procedures. Control sections for single and dual labeling procedures were processed identically with the exception that primary antibodies were omitted from the incubation solution. Single labeling was observed when one of the primary antibodies was omitted, and no staining was detected in the absence of both antibodies.

4.4 Results

Light microscopic dual immunolabeling: D2 (Vector SG) and ChAT (DAB)

Cholinergic interneurons of the dorsal striatum and shell and core compartments of the nucleus accumbens were identified by ChAT-IR visualized by the brown DAB immunoreaction product. ChAT immunoreactive neurons displayed the morphological features characteristic of large aspiny cholinergic interneurons (Kemp and Powell, 1971;

Phelps et al., 1985). The somas of these neurons were oval, elongated, or multipolar, with a 30–50-µm soma diameter from which several, infrequently branching dendrites emerged, extending up to a millimeter in length. Cholinergic interneurons of the dorsal striatum and nucleus accumbens were typically situated in clusters (see Fig. 4.1). The striatal neuropil contained an extensive arborization of immunolabeled cholinergic dendrites and axons.

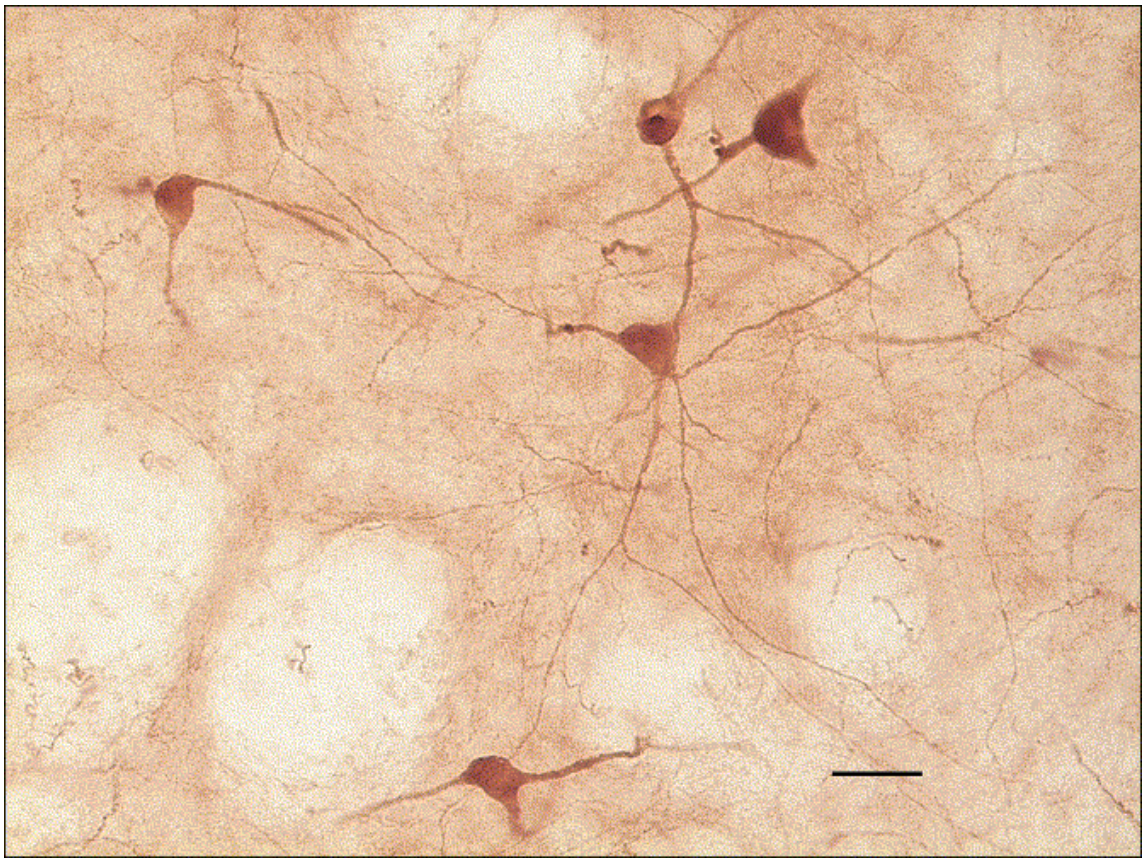

Figure 4.1 Cholinergic Interneuron of the striatum. Cholinergic interneurons located in the striatum of the rat brain were identified by immunocytochemical labeling procedures using an anti-ChAT antibody and DAB label. Cholinergic cell somata measured 30–50 microns in diameter and their neuronal processes measured up to a millimeter in length. The neurons were frequently situated in neuronal clusters. Scale Bar =50 µm.

The areas examined for dopamine D2 receptor and ChAT dual-labeling were contiguous 70 micron-thick coronal sections taken between 2.70 and 0.70 mm anterior to Bregma (Paxinos and Watson, 1998) (see Fig. 4.2). Dual-labeling procedures revealed intense dopamine D2 receptor immunolabeling on striatal cholinergic interneurons located throughout the rostro-caudal extent of the dorsal striatum and nucleus accumbens of the rat. Dopamine D2 receptor labeled cholinergic interneurons in the dorsal striatum are shown in Fig. 4.3. Strong dopamine D2 receptor-IR, indicated by the Vector SG blue label, appeared on the perikarya of the cholinergic neurons and on circumscribed regions of their dendritic processes.

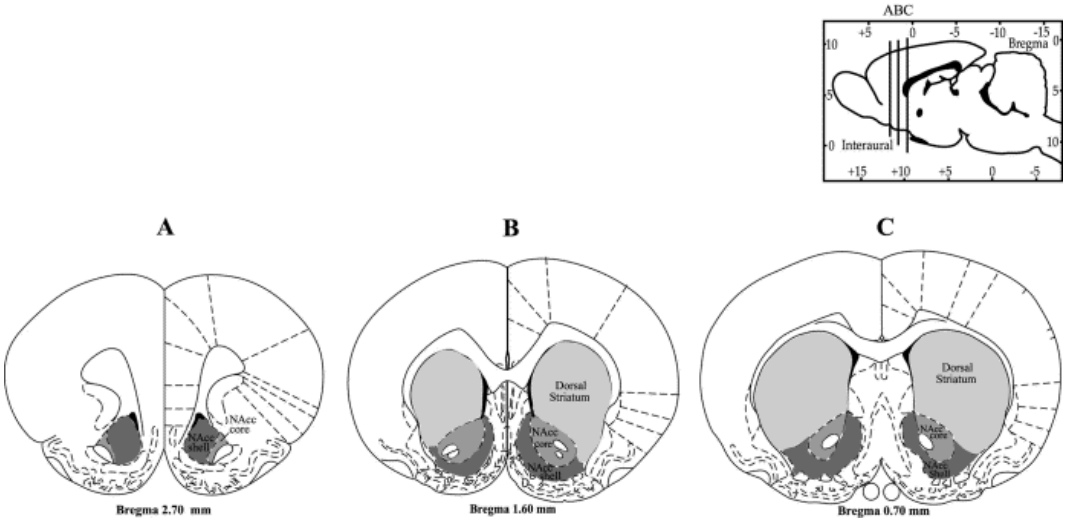

Figure 4.2 Dorsal striatum and nucleus accumbens atlas plates. Coronal sections from the atlas of Paxinos and Watson (1998) illustrate the boundaries of the rostro-caudal extent of the dorsal striatum and nucleus accumbens (Bregma 2.70 mm, Bregma 0.70 mm, A, C) that were investigated in this study. A representative midregion section (Bregma 1.60 mm, B) is also illustrated.

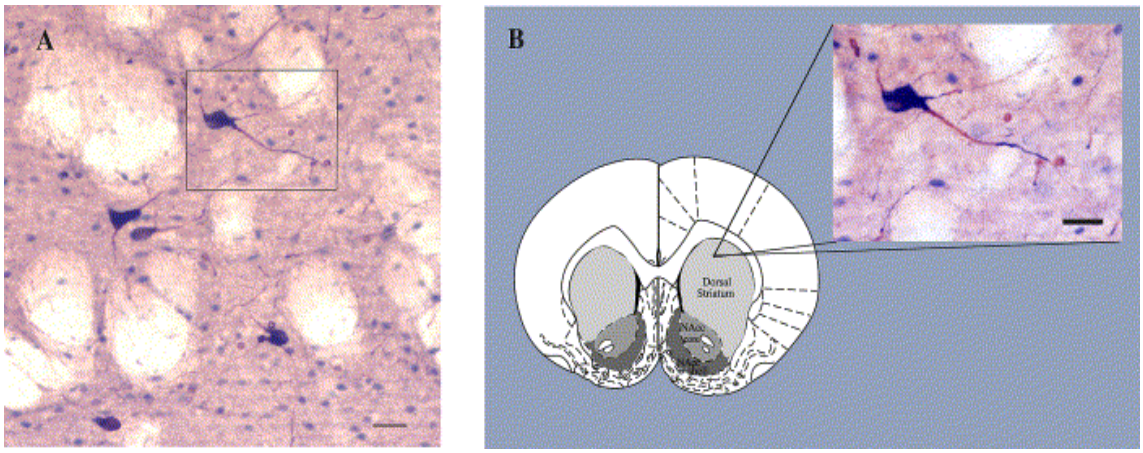

Figure 4.3 Dopamine D2 receptor localization on cholinergic interneurons of the dorsal striatum (A) Dopamine D2 receptors were localized on cholinergic interneurons of the dorsal striatum. Cholinergic interneurons were identified by ChAT-immunoreactivity visualized by the brown DAB label, whereas the dopamine D2 receptor-IR was recognized by the blue SG label. The dopamine D2 receptor was also evident on non-cholinergic neurons, presumably medium spiny output neurons. The dual labeled neuron that is outlined by the box is shown at higher magnification in B. (B) A high magnification light micrograph demonstrates that dopamine D2 receptor-IR (blue-SG label) was present in the cell soma and at circumscribed regions along the processes of a cholinergic interneuron (brown-DAB label) that was located in the dorsal striatum. Scale bars =35 μm.

The cell somata and processes of cholinergic interneurons located in the NAcc also contained the dopamine D2 receptor (Fig. 4.4). Dopamine D2 receptor-IR also appeared on numerous medium-sized neurons in the dorsal striatum and NAcc. Such medium-sized neurons are presumably GABAergic projection neurons and perhaps other striatal interneurons. The number of striatal neurons expressing the dopamine D2 receptor was not analyzed quantitatively. However, qualitative analysis reveals that most cholinergic interneurons in the dorsal striatum and nucleus accumbens display dopamine D2 receptor-IR. The axons of dorsal striatal and accumbal cholinergic neurons also

contained the dopamine D2 receptor. Receptor localization on a cholinergic axon of the NAcc is shown in Fig. 4.5.

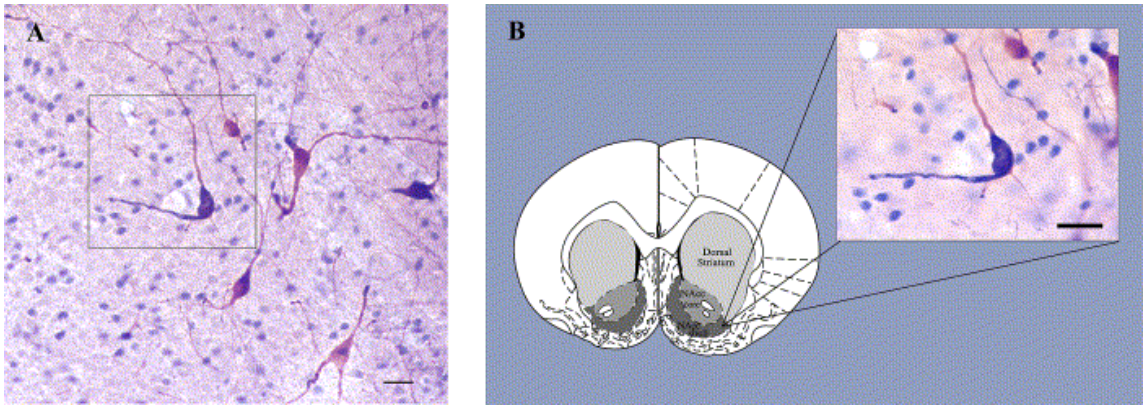

Figure 4.4 Dopamine D2 receptor localization on cholinergic interneurons of the nucleus accumbens. (A) Dopamine D2 receptor-IR (blue-SG label) was evident on ChAT-positive (brown-DAB label) cholinergic interneurons of the nucleus accumbens. ChAT-IR was present in the perikarya and processes of these neurons. Dual-labeled neurons were present in both the shell and core compartments of the nucleus accumbens. The dual labeled neuron that is outlined by the box is shown at higher magnification in B. (B) A high magnification light micrograph demonstrates dopamine D2 receptor-IR (blue-SG label) in the cell soma and circumscribed regions of the processes of a cholinergic interneuron located in the shell compartment of the nucleus accumbens. Scale bars =35 μm.

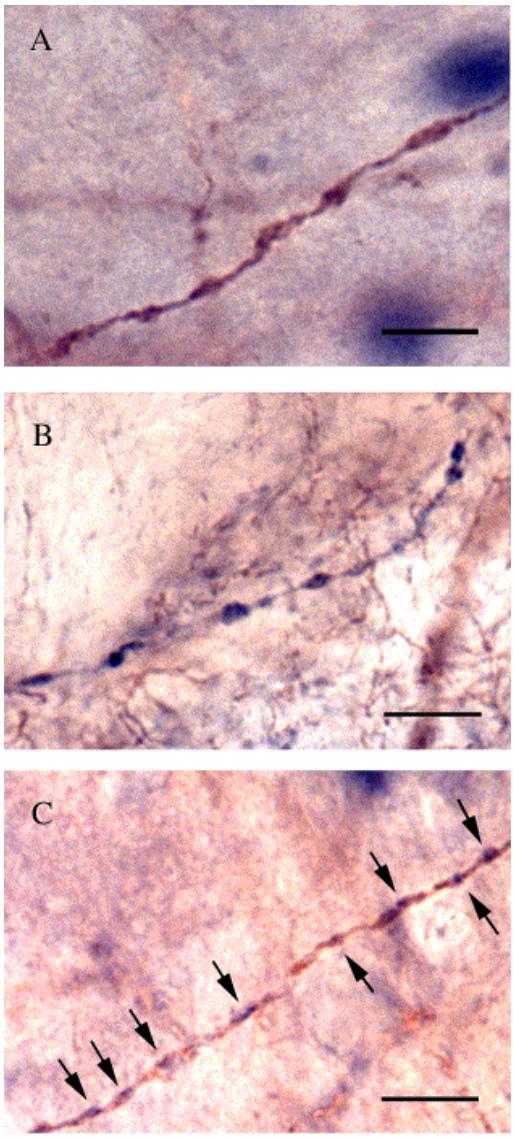

Figure 4.5. Dopamine D2 receptor localization on axons of the nucleus accumbens and dorsal striatum. The dopamine D2 receptor was present on cholinergic axons located in the dorsal and ventral striatum of the rat brain. Cholinergic axons were recognized by a brown DAB label as illustrated in an accumbal cholinergic axon illustrated in A. Dopamine D2 receptor-positive axons in the striatum were identified by the blue-SG label. An accumbal dopamine D2 receptor-IR axon is illustrated in B. Occasionally, the dopamine D2 receptor was observed on cholinergic axons located in the dorsal and ventral striatum. A dual labeled (ChAT/D2) axon of the nucleus accumbens is shown in C. The dopamine D2 receptor is identified as the blue-SG label (arrows) on the brown DAB labeled cholinergic axon. The neuronal processes possess varicosities, which is a typical characteristic of striatal axons. Scale bars =10 μm.

4.5 Discussion

The main finding of this study was the cellular localization of dopamine D2 receptors on cholinergic interneurons of the dorsal striatum and NAcc of the rat brain. Dopamine D2 receptors are targeted by several therapeutic drugs used in the treatment of such striatal-related disorders as Tourette Syndrome, Parkinson's disease, and schizophrenia. Additionally, dopamine D2 receptors play a critical role in relapse and drug seeking behavior associated with several drugs of abuse (Self et al., 1996; De Vries and Shippenberg, 2002). The involvement of the mesolimbic DA pathway in drug abuse has been well documented. More recently, we and others have reported the involvement of cholinergic interneurons of the dorsal striatum and the shell compartment of the NAcc, which are areas targeted by the mesolimbic DA pathway, following the administration of such drugs of abuse as cocaine and alcohol (Consolo et al., 1999; Mark et al., 1999; Berlanga et al., 2003; Herring et al., 2004). The localization of dopamine receptors on dorsal striatal and accumbal cholinergic interneurons should therefore provide a clearer understanding of the cellular mechanisms that underlie addiction and other striatal-related clinical disorders. Ultimately, such findings should lead to the development of improved site-specific targeted behavioral and pharmaceutical treatments for these disorders.

The cellular localization of dopamine D2 receptors on cholinergic interneurons of the dorsal striatum and nucleus accumbens

The present study revealed that dopamine D2 receptors were localized on the perikarya, dendrites, and axons of cholinergic interneurons in the dorsal striatum and nucleus accumbens of the rat. These data are in accordance with evidence that the dopamine D2 receptor mRNA is present in striatal cholinergic interneurons (Surmeier et al., 1993; Yan et al., 1997; Nicola et al., 2000) and with pharmacological evidence

suggesting a direct dopamine D2 receptor effect on cholinergic interneurons of the striatum (Gorell and Czarnecki, 1986). Specifically, dopamine D2 receptors attenuate the release of acetylcholine release (Lehmann and Langer, 1983; Stoof et al., 1987; Bertorelli and Consolo, 1990; Damsma et al., 1990; Drukarch et al., 1990) by reducing N-type Ca2+ currents via a membrane-delimited, Gi/o class G-protein pathway (Yan and Surmeier, 1997). Co-activation of the dopamine D1 and D2 receptors typically produce opposing effects. The D2 receptor inactivates the D1 receptor/cAMP/DARPP-32/PP1 pathway by inhibiting cAMP or alternatively by activating PP-2B and dephosphorylating DARPP-32 (Greengard, 2001). The co-activation of the D1 and D2 receptors, however, can also produce synergistic effects (LaHoste and Marshall, 1993; Hu and White, 1994). The separate or combined effects of DA D1 and D2 receptors on cholinergic neurons can therefore result in a range of cellular and behavioral effects.

Medium-sized neurons, presumably the GABAergic output neurons of the striatum, were also observed to express dopamine D2 receptors, which is consistent with previous reports (Yung et al., 1995). Interestingly, ultrastructural evidence has demonstrated that dopamine D2 receptors on MSNs are relatively more localized on dendrites of the MSNs in the dorsal striatum and more on the axons of these neurons in the NAcc (Delle Donne et al., 1997). The behavioral outcome of DA D2 receptor activation will ultimately depend on the precise cellular localization of the receptor. For example, GABA release from medium spiny neurons is inhibited through the activation of the dopamine D2 receptor. Attenuation of this GABAergic influence onto cholinergic neurons causes the facilitation of acetylcholine release. Alternatively, the activation of dopamine D2 receptors on cholinergic interneurons will attenuate the release of acetylcholine. Glutamatergic corticostriatal and dopaminergic striatal afferent fibers have also been reported to possess dopamine D2 receptors (Sesack et al., 1994; Wang and

Pickel, 2002). The effects of dopaminergic signaling in the striatum are therefore widespread due to the presence of dopamine receptors on the dendrites and axons of a variety of striatal neurons including cholinergic interneurons and on striatal axonal afferent pathways.

Dopamine D2 receptor involvement in addiction and other striatal-related clinical disorders

Treatments for a variety of clinical disorders have been developed which specifically target the dopamine D2-class receptors. Haloperidol and clozapine, which are used in the treatment of Tourette's syndrome and schizophrenia, respectively, are dopamine D2 receptor antagonists. Alternatively, bromocriptine, which is used to treat Parkinson's disease, is a dopamine D2 receptor agonist. Dopamine D2 receptors specifically play an important role in reinstated drug-seeking behaviors, whereas dopamine D1 receptors are more critical to drug-induced satiation and tolerance (Self et al., 1996). More specifically, dopamine D2 receptor family agonists have been shown to mimic the priming effects of cocaine in monkeys trained to self-administer the drug, whereas dopamine D1 receptor agonists did not reinstate the behavior (Khroyan et al., 2000). In addition to the importance of DA D2 receptors in relapse and the reinstatement of drug-seeking behaviors, (Self et al., 1996; De Vries and Shippenberg, 2002), these receptors have also been reported to be up-regulated during early withdrawal (Sousa et al., 1999). These receptors and their respective cellular microcircuits may therefore prove to be critical target sites for drug treatment programs.

Moreover, the dopamine D2 receptor activation on cholinergic interneurons may oppose the effects of DA D1 receptors or alternatively may play a unique role in potentiating the effects of ACh release through the synergistic effects of combined D1

and D2 activation (LaHoste and Marshall, 1993; Hu and White, 1994). Earlier in situ hybridization studies reported the presence of dopamine D1 and D2 receptor mRNAs on separate populations of striatal projection neurons (Gerfen et al., 1990; Gerfen et al., 1995), however more recent findings report D1- and D2-receptor co-localization using immunocytochemical methods, (Surmeier et al., 1993; Gerfen and Keefe, 1994). Our data are in good agreement with previous in situ hybridization studies, which report D1 and D2 mRNA on cholinergic neurons (Nicola et al., 2000) as well as recent immunocytochemical work that found D1-class receptor localization on cholinergic neurons. Dopamine D1-class receptors, specifically the D5 receptor subtypes, which have an affinity for dopamine ten times higher than that of D1 receptors (Grandy et al., 1991), have recently been localized on striatal cholinergic interneurons of the nucleus accumbens and other brain areas (Rivera et al., 2002; Berlanga et al., 2005). Virtually all cholinergic interneurons in the dorsal striatum and NAcc express the D5 receptor. It is likely that the combined effects of D1/D5- and the D2-class receptors (opposing or synergistic), which we have localized on cholinergic interneurons in the present study, will depend on the specific pharmacological and behavioral stimuli and respective microcircuits that elicit their activation. As a result, the overall cellular and behavioral effects of a drug may differ depending on whether the drug is self-administered or administered passively, see for example, (Mark et al., 1999).

Conclusions and implications for future studies

In conclusion, DA–ACh interactions are critical for striatal function and pivotal to a variety of clinical disorders. Dopaminergic signaling occurs through the activation of D1- and D2-class receptors. The localization of these receptors has been reported on medium spiny output neurons as well as on striatal glutamatergic and dopaminergic

afferent projections. This study is the first to report the cellular localization of dopamine D2 receptors on cholinergic interneurons. The D1-class receptor subtype, D5, has also recently been reported to be localized on accumbal cholinergic interneurons. Of critical importance will be to co-localize D5 and D2 receptors on these cholinergic neurons since their potential synergistic effects may be critical to such behavioral phenomenon as learning (Calabresi et al., 1992). It will also be important to examine, in future studies, the effects of converging DA and glutamate inputs onto these cholinergic neurons since such convergence may underlie associative learning, which is posited to play an important role in addiction (Vanderschuren and Kalivas, 2000; Anagnostaras et al., 2002).

Our findings should provide a basis for future investigations designed to better understand the DA influence onto cholinergic neuronal circuitry of the dorsal striatum and nucleus accumbens. Such knowledge should contribute to our understanding of the underlying molecular basis of addiction and other clinical disorders. Focus on the cholinergic system and its interface with the DA mesolimbic pathway and glutamatergic limbic and prefronto-striatal pathways should identify microcircuits that should be targeted by pharmaceutical and behavioral treatment programs for addiction and a variety of motor and neuropsychiatric disorders.

Chapter 5: Morphine- and Cocaine-Induced Synaptic Rewiring in the Nucleus Accumbens: Potential Neural Substrates Underlying Drug-Induced Behavioral Changes

5.1 Abstract

Repeated use of psychostimulants and opiates can lead to such behavioral changes as motor sensitization, tolerance, psychosis and relapse. Many studies have attempted to identify molecular and cellular events responsible for such drug-induced behaviors, which can persist for years. Increasing evidence suggests that these behavioral changes may be mediated by persistent structural changes in the brain, such as changes in synapse number. A major site of synaptic contact is the dendritic spine (Harris and Kater, 1994). Previous studies have reported an increase in the density of spines on medium spiny neurons of the nucleus accumbens (NAcc) and pyramidal cells of the prefrontal cortex (PFC) following repeated injections of cocaine (Robinson and Kolb, 1999b), and a decrease in spine density following morphine injections (Robinson and Kolb, 1999a). In a more recent study, motor sensitization was associated with an increase in spine density in the core compartment of the NAcc of cocaine-sensitized animals (Li et al., 2004). Evidence of such morphological changes in the NAcc following long-term drug intake suggested that synaptic changes were also occurring. Therefore, the present study tested the hypotheses that the synapse per neuron ratio would increase in the NAcc shell and core of cocaine-treated rats and decrease in morphine treated animals, and that increases in the number of synapses per neuron in the NAcc core would be associated with animals exhibiting behavioral sensitization.

Adult female Sprague-Dawley rats were given 20 days of repeated, intermittent injections of either 15 mg/kg cocaine or 10 mg/kg morphine. Animals were left

undisturbed for an additional 3 weeks to test for the persistence of structural changes. Electron microscopy and unbiased quantitative stereological analyses revealed an increase in the synapse per neuron ratio in the NAcc shell of cocaine-treated (49.1%) and morphine-treated (55.1%) rats, relative to controls. However, an increase in the synapse per neuron ratio in the NAcc core was found only in cocaine-sensitized animals (49.1%). More specifically, the increases found in both the shell and the core, were primarily due to an increase in the number of asymmetric (Gray's Type I) synapses, suggesting plasticity in glutamatergic synapses. This study provides the first ultrastructural evidence of cocaine-induced synaptic changes, and identifies functionally distinct types of synapses that underlie cocaine-induced motor sensitization.

5.2 Introduction

Recent studies indicate that long-term use of drugs of abuse leads to neuroadaptations in the nucleus accumbens (NAcc) at the level of gene expression (Carlezon et al., 1998; Shaw-Lutchman et al., 2002) and at the level of cellular morphology (Robinson and Kolb, 1999a, b; Norrholm et al., 2003). Such changes presumably underlie long-lasting drug-induced behaviors observed during escalated drug intake and after prolonged abstinence from addictive substances (Chao and Nestler, 2004).

Long-term treatment with cocaine and morphine induce structural changes in the NAcc shell and medial PFC, two primary targets of the mesolimbic dopamine (DA) pathway. Golgi-Cox staining of output neurons in these brain areas reveals an increase in dendritic branching and spine density following repeated i.p. treatment with cocaine (Robinson and Kolb, 1999b; Norrholm et al., 2003) and a decrease in dendritic branching and spine density following repeated i.p. treatment with morphine (Robinson and Kolb, 1999a). Furthermore, spine density changes in the NAcc core have recently been

correlated with the expression of behavioral sensitization (Li et al., 2004). Thus, the spine density changes detected by Robinson and colleagues may underlie the persistent behavioral changes observed even after drug cessation.

At the behavioral level, sensitization and tolerance reportedly occur following repeated cocaine and morphine treatment. The expression of behavioral sensitization and tolerance varies depending on the drug dose and frequency of administration. In general, cocaine-sensitized rats exhibit enhanced exploratory behaviors such as sniffing, locomotor activity and rearing, as well as stereotyped sniffing and headbobbing minutes after drug administration (Wise, 1984; Guan et al., 1985; Zahniser et al., 1988; Steketee et al., 1992; O'Dell et al., 1996). Morphine-sensitized rats also demonstrate an enhanced locomotor response to repeated drug administration (Jeziorski and White, 1995; Cadoni and Di Chiara, 1999). Furthermore, prolonged treatment with morphine leads to the expression of a stereotyped chewing/gnawing behavior (Pollock and Kornetsky, 1989; Kornetsky, 2004). Alternatively, behavioral tolerance in cocaine-treated rats is not as readily detectable as in morphine-treated animals. Continuous administration of cocaine (5 to 40 mg/kg per day) over the course of 14 days has been shown to induce behavioral tolerance (King et al., 1999), whereas behavioral tolerance to the analgesic effects of morphine, if administered continuously (16.8 to 36 i.v. mg/kg per day), can develop within 8 to 12 hours (Ouellet and Pollack, 1995) or after 7-10 days if administered 10 mg/kg once a day intraperitoneally (Yamamoto et al., 1988). Examination of specific microcircuits and drug-induced synaptic changes underlying these drug-induced behaviors require further examination.

The structural alterations in reward and motor related brain regions that accompany the observed behavioral changes resulting from repeated treatment with cocaine and morphine suggest that synaptic reorganization may also be occurring. Such

synaptic changes have not been previously examined. The spine density changes observed using the Golgi-Cox staining procedure and the assumption that spine density changes extend to synaptic changes has long been open to criticism. For example, this staining technique only stains a small percentage (approximately 1-5%) of the total neurons (Pasternak and Woolsey, 1975). Therefore, synaptic changes associated with long-term drug treatment must be confirmed through direct examination at the electron microscopic level. Evidence of changes in synapse number will require future studies at the electron microscopic level to provide insight into the specific types of synapses that are changing. The present study tested the hypothesis that the number of synapses per neuron increase in the NAcc shell and core of cocaine and morphine treated rats.

5.3 Methods

All experimental manipulations were carried out during the light portion of the cycle. Animals were handled for 15 minutes twice daily and vaginal smears were obtained once daily for 7 days prior to any experimental manipulation. Experimental procedures conformed to National Institutes of Health guidelines and were carried out under an institutionally reviewed and approved research protocol.

5.3.1 Animals

Twenty-four adult female Sprague-Dawley rats weighing 250-300g, provided by the Animal Resources Center at the University of Texas at Austin, were used in this study. Rats were individually housed in clear plastic hanging cages (45 x 23.5 x 20.75 cm) in an animal colony room with ad libitum access to food and water, under a 12 hour light-dark cycle (lights on at 0700 hours). Daily vaginal smears were collected prior to drug injections at approximately the same time every day during the 4 week drug treatment. Two animals had to be removed from the study. One received the wrong drug

injection on the last testing day, and another developed a growth near the site of injection after the first day of testing. All experimental manipulations were carried out during the light portion of the cycle. Animals were handled for 15 minutes twice daily and vaginal smears were obtained once daily for 7 days prior to any experimental manipulation. Experimental procedures conformed to National Institutes of Health guidelines and were carried out under an institutionally reviewed and approved research protocol.

5.3.2 Drug Administration

Rats were randomly divided into 3 groups, Cocaine (n=7), Morphine (n=7), and Saline (n=8). Morphine sulfate (10 mg/kg) and cocaine hydrochloride (15 mg/kg) were dissolved in 0.9% saline and injected i.p. at 1 mL/kg of body weight. All doses are expressed as the weight of the salt. Drug injections were administered in the testing room and animals were immediately placed into the test cage following the injection. The test cage was a clear plastic cage (45 x 23.5 x 20.75 cm). Grid lines were made on the outside of the bottom of the cage dividing the cage into 6 quadrants. The lines were used to score line crosses following drug administration. On the first day of drug testing, rats were removed from their home cage and were given an i.p. injection of 15 mg/kg cocaine hydrochloride, 10 mg/kg morphine sulfate, or 1 mL/kg of 0.9 % saline followed by confinement to the test cage. Motor activity in the test cage was recorded with a video camera for 60 minutes immediately following the injection. Animals were then returned to their home cage following the testing session. These procedures were repeated 5 days a week, followed by 2 consecutive drug-free days over a total of 4 weeks. After the last testing day, animals were left undisturbed for an additional 21 days before their brains were removed.

5.3.3 Behavioral Analysis

Immediately following the drug injection, animals were placed into the testing cage and their behavior was recorded for 60 minutes. Video tapes were coded and later viewed by observers blind to group assignment. Behaviors quantified from the video footage included: line crosses, rearing, headbobbing, sniffing, grooming, immobility, chewing/gnawing, and sleeping. Line crosses were counted when both forepaws crossed a line as the rat moved in the forward direction. Rearing activity was measured when the rat stood on its hind legs, either supported by the cage or unsupported. Headbobs were counted as head movements in the vertical direction (up to down), while stationary with all paws on the ground. Sniffing was timed while the rat was in a rearing position (sniffing up) or a standing position with all four paws on the ground (sniffing down). Grooming behavior was timed for only grooming of the face and head. Immobility was characterized by freezing behavior in the absence of head movement. Chewing/gnawing behavior was recorded when animals chewed either their claws (forepaws or hind paws) or the walls of the cage. Sleeping behavior was timed when rats were immobile with their eyes closed or when they tucked their heads under the trunk of their body, sometimes with their tail wrapped around them. Grooming, sniffing, immobility, chewing/gnawing and sleeping were recorded measured in the number of bouts and in the number of seconds.

5.3.4 Tissue Preparation

Animals were administered an overdose of sodium pentobarbital (Nembutal; 100 mg/kg i.p.) and perfused transcardially with 60 ml of 0.1 M phosphate buffered saline (PBS), pH 7.4 followed by 200 ml of 4% paraformaldehyde/0.6% glutaraldehyde in PBS, pH 7.4. The brains were removed and post-fixed for 2 hours in 4% paraformaldehyde/0.6% glutaraldehyde in PBS. Coronal sections were taken at a

thickness of 100 μm on a vibratome. Free-floating sections were then processed for electron microscopy. All efforts were made to minimize animal suffering and the number of animals used.

5.3.5 Electron Microscopy

The tissue sections were rinsed in 0.1M phosphate buffer (PB) and incubated in 1% osmium tetroxide in PBS for 1 hour. Tissue is dehydrated through a graded series of alcohol concentrations, propylene oxide, and a 50:50 mixture of propylene oxide and Embed-812 (Electron Microscopy Sciences). The tissue sections were placed on an open rotator overnight in 100% Embed-812 (medium hardness). The following day, specimens were placed in fresh epon and flat embedded between two sheets of ACLAR film (Electron Microscopy Sciences) and cured at 60°C for 48 hours. Polymerized and osmicated tissue sections were inspected under a light microscope to locate the target region using reliable neuroanatomical landmarks corresponding to the Paxinos and Watson Stereotaxic Coordinates for the Rat Brain (1995). The target region was excised and then glued to an epon rod, one from the NAcc shell and one from the NAcc core. A glass knife was used to trim the specimen to a size of 0.5 by 0.5 mm on an ultramicrotome (Leica Ultracut UCT). One block of tissue was used for each animal for each brain region. Ten semithin sections (~500 nm-thick) and four ultrathin sections (~70 nm-thick) were cut on the ultramicrotome using a diamond knife (Ted Pella) from the same block of tissue from each animal. Every other semithin section was dried onto a superfrosted glass slide (5 sections), at which point a ribbon of 4 serial ultrathin sections was collected onto a formvar-coated slot grid. This process was repeated four times until a total of 20 semithin sections were collected onto a glass slide and 16 ultrathin sections were collected onto slot grids (4 slot grids total). Semithin sections were stained with toluidine blue and ultrathin sections were counterstained with 2% uranyl acetate

(aqueous) and examined and photographed using a Philips EM208 transmission electron microscope (TEM) (FEI Company) at 80kv. Images, 1 Mb in size, were collected with an AMT Advantage HT camera.

5.3.6 Electron Microscopic Data Analysis

All photographs were taken at a magnification of 18000X. Images were stored and analyzed by one observer blind to group assignment. The observer counted all synapses in the shell and core compartments of the NAcc. Synapses were categorized as asymmetric, symmetric or undefined. Asymmetric synapses were identified by the presence of at least three round synaptic vesicles in the presynaptic terminal and a postsynaptic density, and symmetric synapses were identified by the presence of three flat (i.e. pleomorphic) vesicles and two closely apposing membranes. The postsynaptic target was identified for each synapse quantified in the study whenever possible.

5.3.7 Stereological Methods

The present study examined changes in the synapse per neuron ratio because this ratio has been shown to more accurately reflect changes in synapse number when neuron number is stable (Anker and Cragg, 1974). Furthermore, tissue shrinkage effects contributed equally to synapse density and neuronal density because measurements of neuronal and synaptic density are obtained from the same epon-embedded samples. An unbiased stereological estimation of the total number of synapses called the physical disector method was used to calculate neuronal density and synapse density, which involves comparing two serial sections (Gundersen et al., 1988). The first section was called the reference section and the second was the look-up section. When the object of interest was found in the reference section but not the look-up section, the object was counted.

Neuron Density

Ten 500 nm-thick sections, described above, were used for neuron density analysis. Each semithin section was stained with toluidine blue, and cells were identified at the light level using a 40X lens on a computer assisted- light microscope. The physical disector method was used to calculate neuronal density, which involves comparing two serial sections. Within an unbiased counting frame of a known area (A_{frame}), the number of nuclei that were present in the reference section and not in the look-up section was counted (Q^-_{neuron}). Next, the disector volume of tissue (V_{dis}) is calculated using the following formula:

$$V_{dis} = A_{frame} \times H \qquad \text{(i)}$$

where H is section thickness (1 μm) multiplied by the number of sections. The neuronal density, $N\nu_{neuron}$, was then determined by dividing the number of cells counted by the volume of the tissue using the following formula:

$$N\nu_{neuron} = Q^-_{neuron} / V_{dis} \text{ (using semithin sections)} \qquad \text{(ii)}$$

Synapse Density

The ultrathin sections collected on formvar-covered copper slot grids were stained with 2% aqueous uranyl acetate before viewed under the TEM. Twenty micrographs were taken from two adjacent sections (~ 70 um thick) in the same position for a total of

40 micrographs per animal (twenty disector pairs), using one slot grid. The physical disector method was again used to calculate the synapse density, for which the number of synapses present in the reference section and not the look-up section were counted ($Q^-_{synapse}$). The total number of synapses within an unbiased counting frame of a known area (A_{frame}) was counted. Again, the disector volume of tissue (V_{dis}) is calculated using formula (i) from above, where H is section thickness (70 nm) multiplied by the number of sections. Synapse density, $N_{V_{synapse}}$, was calculated using the following formula:

$$N_{V_{synapse}} = Q^-_{synapse} / V_{dis} \text{ (using ultrathin sections)} \quad \text{(iii)}$$

The number of synapses per neuron was determined by dividing the density of synapses per cubic millimeter by the neuronal density per cubic millimeter, as shown below:

$$N_{V_{neuron}} / N_{V_{synapse}} \text{ (synapse to neuron ratio)} \quad \text{(iv)}$$

Section thickness of ultrathin sections was verified using "Small-Fold's" technique (Weibel, 1979). For this calculation an image of a small fold (as compared to a useless fold as described by Weibel, 1979) is captured from an ultrathin section and the width of the fold can be estimated using the image scale bar. The following formula is then used:

$$2t = \ell \quad \text{(v)}$$

where ℓ is the width of the small fold (using the scale bar) and t is the estimated thickness of the ultrathin section.

5.3.8 Statistical Analysis

The statistical analyses of behavioral and anatomical data were designed to test whether 1) rats show behavioral sensitization or tolerance to repeated drug treatment by comparing drug effect on day 1 to day 20 and 2) whether accompanying changes in the synapse per neuron ratio in the NAcc shell and core are significantly different in the drug-treated animals relative to control animals. Statistical analyses of behavioral data were performed using SPSS (SPSS, Inc.) repeated-measures analysis of variance (ANOVAs) for the effects of Groups, Days, and Group by Day interaction followed by pairwise comparisons using Bonferroni's correction. Significant changes in the synapse per neuron ratio were determined by a one-way ANOVA followed by pairwise comparisons using Bonferroni's correction. Significance level was set at $p < .05$.

5.4 Results

Behavioral Analyses of Non-Stereotypic Behaviors. As expected, cocaine-treated animals showed significantly more locomotor activity (line crosses and rearing) relative to control animals both on days 1 and 20 (Fig. 5.1A, B). A significant decrease in the total number of line crosses (Fig. 5.1A) and rears (Fig. 5.1B) was observed in cocaine-treated animals by day 20. In contrast to cocaine, morphine administration on day 1 did not produce significant increases in locomotor activity relative to controls (Fig. 5.1A, B). Instead, morphine administration on day 1 produced motor inhibition (opiate catalepsy), reported here as immobility (Fig. 5.1C). Furthermore this drug-induced immobility was significantly higher on day 1 relative to day 20, suggesting evidence of motor tolerance.

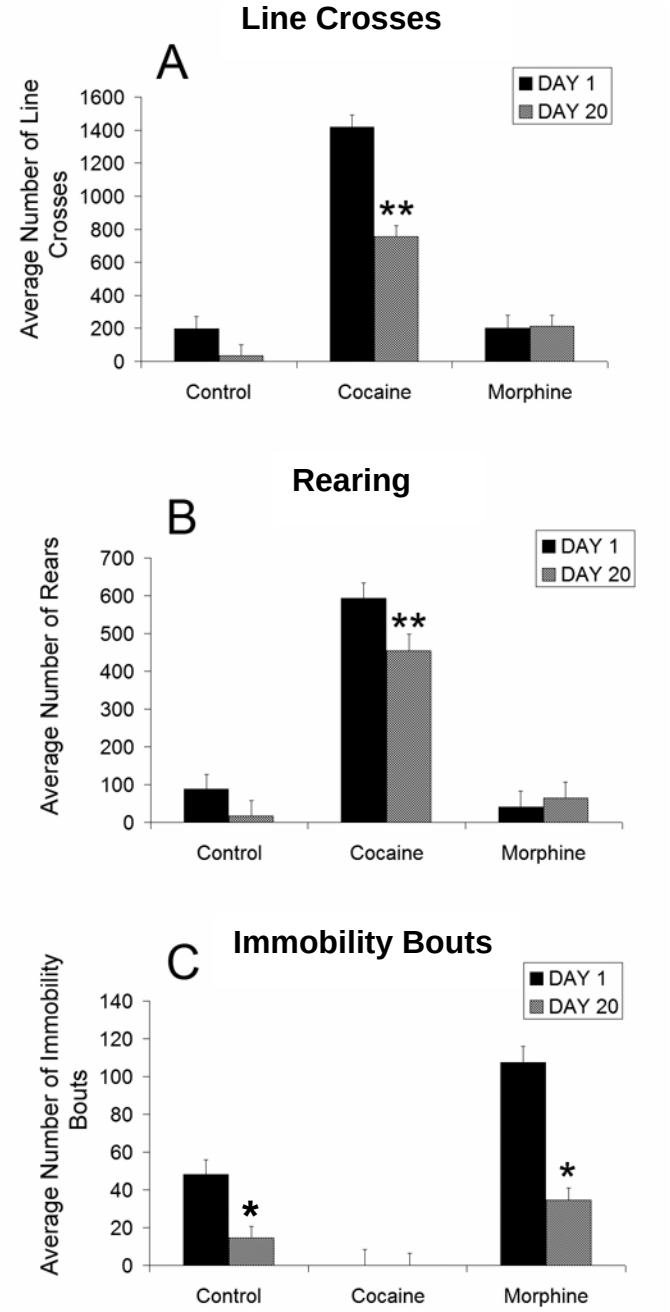

Figure 5.1. Non-stereotypic behaviors. The average (±SEM) number of line crosses (A) rears (B) and immobility bouts (C) during the 1-hour testing session, as a function of treatment and day. (** indicates statistical significance ($p < .05$) relative to control group and day 1; * indicates statistical significance ($p < .05$) relative to day 1).

Behavioral Analyses of Stereotypic Behaviors. A significant increase in the average number of stereotypic headbobs (Fig, 5.2A) and stereotypic sniffing (Fig. 5.2B), two reliable measures of cocaine-induced motor sensitization, was found in cocaine-treated animals by day 20 relative to controls. Morphine-treated rats did not exhibit significant behavioral levels of stereotypic headbobbing; however, a pronounced morphine-induced stereotypic chewing/gnawing behavior was evident on day 20 (Fig. 5.2C), also evidence of sensitization.

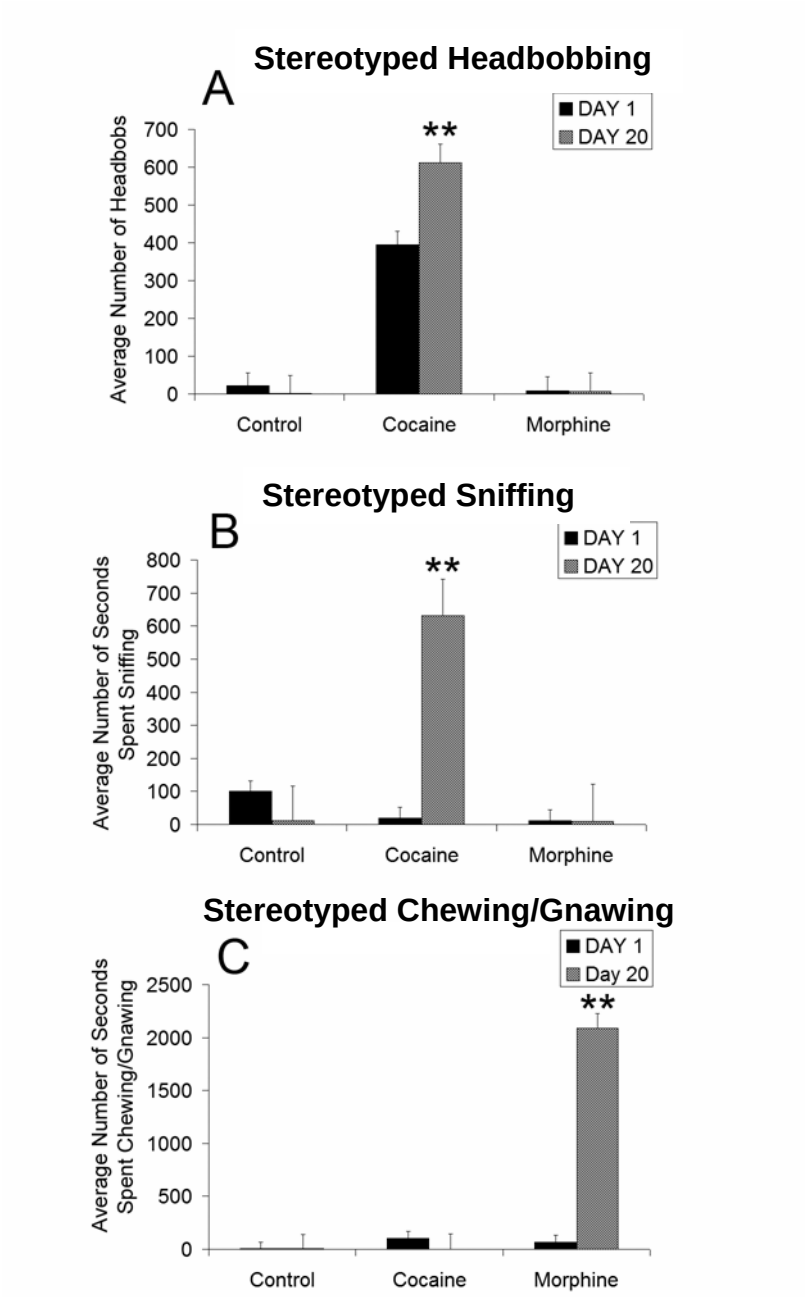

Figure 5.2. Stereotypic behaviors. The average (±SEM) number of headbobs (A) seconds spent sniffing (B) and seconds spent chewing/gnawing during the 1-hour testing session (C), as a function of treatment and day. (** indicates statistical significance ($p < .05$) relative to control group and day 1; * indicates statistical significance ($p < .05$) relative to day 1).

Behavioral Analyses of Sleeping and Grooming Behaviors. Analysis of sleeping behavior revealed that control animals spend significantly more time sleeping on day 20 relative to the first day of testing (Fig. 5.3). Drug-treated animals did not exhibit sleeping behavior during the 1 hour testing session. Therefore, all behavioral measures were adjusted for sleeping behavior observed in the control animals and re-evaluated (data not shown). All statistically significant behaviors reported above, remained significant. Additionally, when grooming behavior was adjusted for the time animals were asleep on day 20, instead of the total 60 minute video, a significant increase in grooming bout duration (i.e. the number of seconds spent grooming per bout) (Fig. 5.4A) and the total number of grooming bouts (Fig. 5.4B) was found when day 1 was compared with day 20.

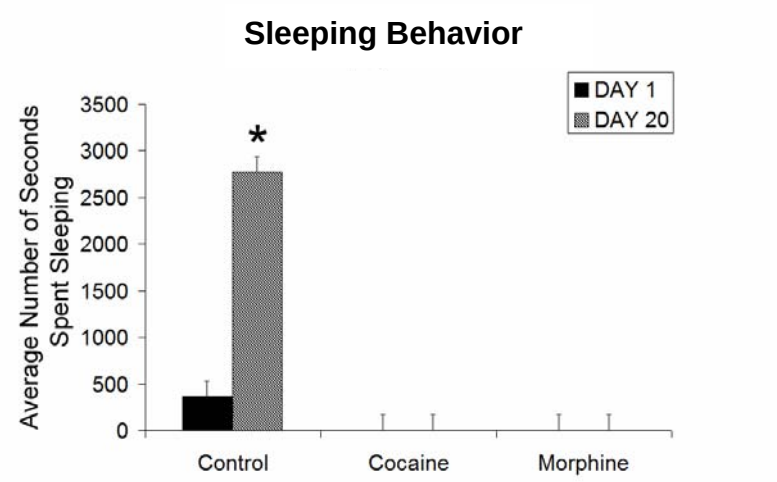

Figure 5.3. Sleeping behavior. The average (±SEM) time (seconds) observed for sleeping behavior, as a function of treatment and day. (* indicates statistical significance ($p < .05$) relative to day 1).

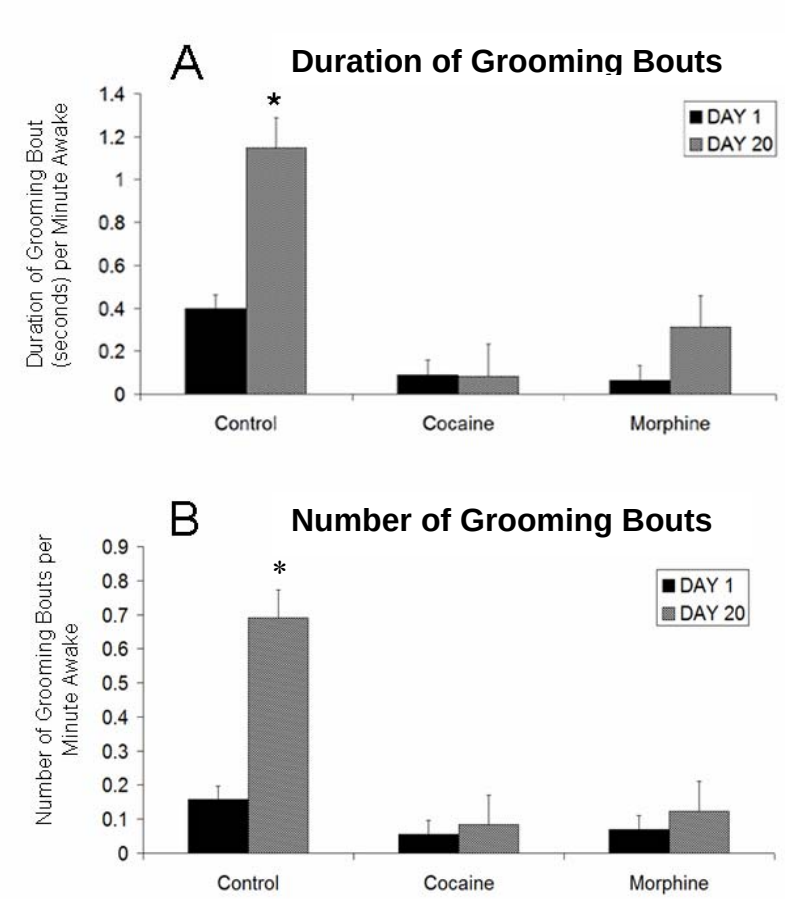

Figure 5.4. Grooming behavior adjusted for time awake. The average (±SEM) number of grooming bouts (A) and average (±SEM) time (seconds) spent grooming (B) per minute awake, as a function of treatment and day. (* indicates statistical significance ($p < .05$) relative to day 1).

Synaptic Quantification NAcc Shell. A significant increase in the total number of synapses per neuron in the shell of cocaine- (49.1%, p = .0.032) and morphine- (55.1%, p = .015) treated animals was found, relative to controls (Fig. 5.5A). Neuron density was stable in all three groups (i.e. not statistically different) (cocaine = 244,113 ± 17,482.699 neurons per mm^3; morphine = 265,175 ± 17,482.699 neurons per mm^3; control = 279,215 ± 16,353.568 neurons per mm^3). Thus, the calculated number of synapses per neuron

reflects a change in the number of synapses. Specifically, there was a significant increase in the number of asymmetric synapses onto cells in the NAcc shell in cocaine-treated animals (62.2%) and morphine-treated animals (64.2%) (Fig. 5.5B). A subsequent correlation analysis was used to determine whether a direct correlation existed between the degree of sensitization (calculated as the difference between the times spent chewing/gnawing on the first and last day of drug treatment) and the number of asymmetric synapses quantified in this brain region. This analysis revealed that a direct negative correlation ($R^2 = 0.7332$) existed between the degree of sensitization and the number of asymmetric synapses per neuron quantified from the NAcc shell of morphine-treated animals (Fig. 5.6). Cocaine-treated animals did not show a direct correlation between synapse number in the NAcc shell and degree of sensitization using either of the stereotypic behaviors where evidence of sensitization was found.

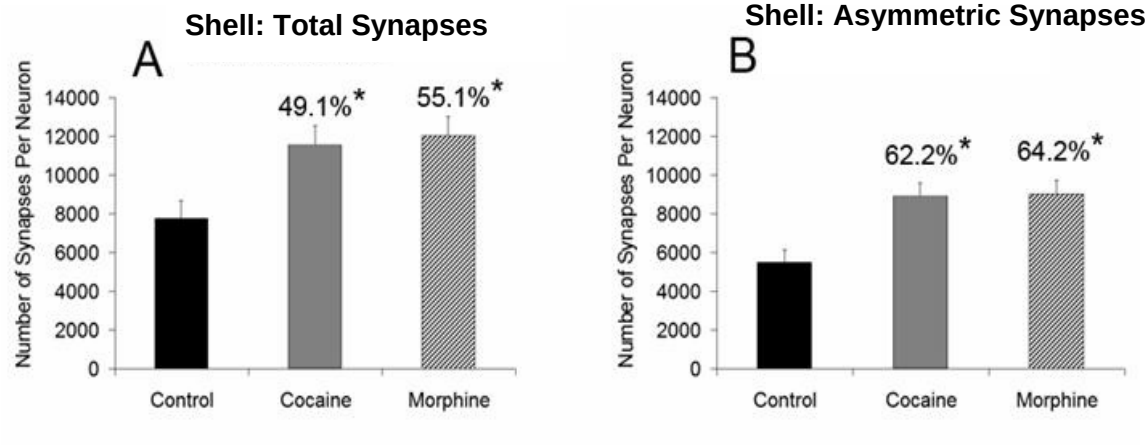

Figure 5.5. Asymmetric synaptic quantification in the NAcc shell. The average (±SEM) number of total synapses (A) and asymmetric synapses (B), as a function of treatment in the NAcc shell. (* indicates statistical significance ($p < .05$) relative to control group).

Synaptic Quantification NAcc Core. A significant increase in the total number of synapses per neuron was also found in the NAcc core of cocaine-treated animals (49%, p

= .013), but not morphine-treated animals, relative to controls (Fig. 5.7A). Again, a stable neuron density (cocaine = 208647 ± 15641.765 neurons per mm^3; morphine = 209167 ± 15641.765 neurons per mm^3; control = 203962.13 ± 14631.531 neurons per mm^3) reveals that this effect is driven by a change in synapse number. In the NAcc core, the number of asymmetric (excitatory) synapses increased by 52.5% ($p = .022$) relative to the control group (Fig. 5.7B). Again, a correlation analysis was used to determine whether a direct correlation existed between the degree of sensitization and the number of asymmetric synapses quantified in the NAcc core. This analysis revealed a positive correlation ($R^2 = 0.7141$) between the degree of sensitization of morphine-treated animals and the number of asymmetric synapses per neuron for the NAcc core (Fig 5.8). No such correlation was found in the NAcc core of cocaine-sensitized animals (data not shown).

The percentage of asymmetric synapses made onto dendritic shafts (Fig. 5.9) and synapses made onto dendritic spines (Fig. 5.10) are reported in Table 5.1, as well as symmetric (inhibitory) synapses (Fig. 5.10), even though no conclusions were drawn from this category of synapses due to the limitations of the sampling strategy, which omitted axosomatic synapses and synapses proximal to the soma, designed for the present study.

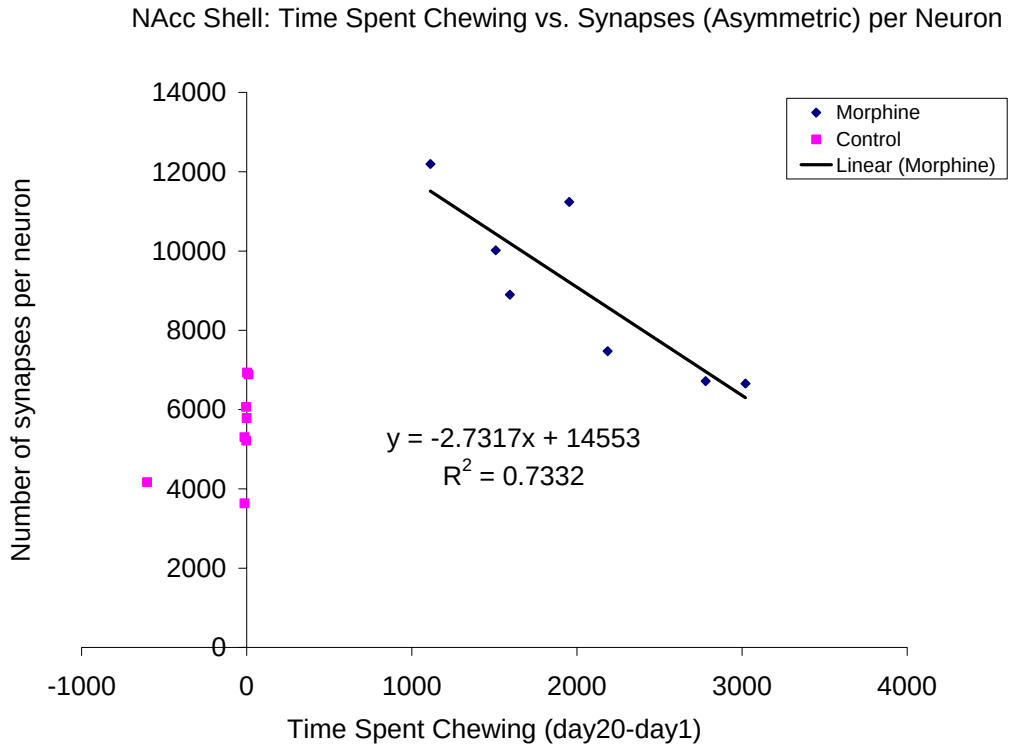

Figure 5.6. Time spent chewing versus the number of asymmetric synapses per neuron in the NAcc shell of morphine-sensitized animals. A correlation analysis suggests a direct negative correlation ($R^2 = 0.7332$) between the number of asymmetric synapses per neuron in the NAcc shell and the degree of sensitization (the difference in the time spent chewing between the last and first day of drug administration).

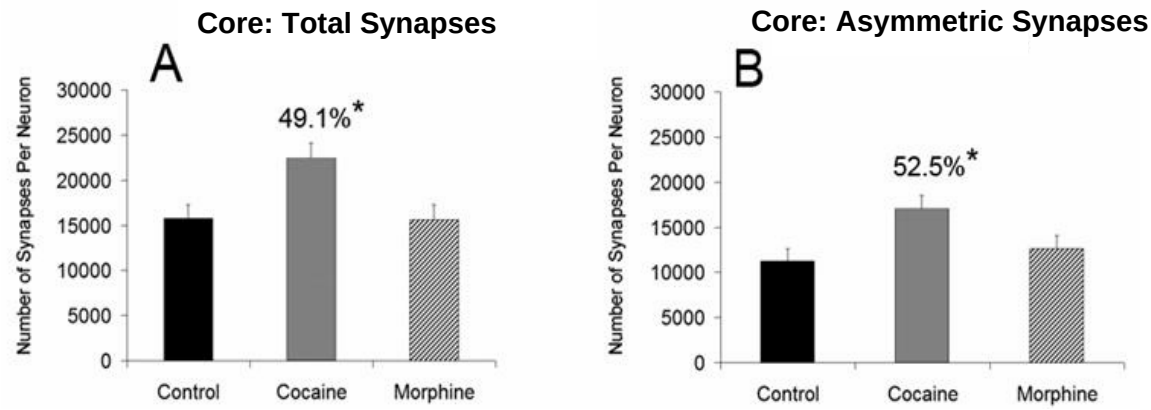

Figure 5.7. Asymmetric synaptic quantification in the NAcc core. The average (±SEM) number of total synapses (A) and asymmetric synapses (B), as a function of treatment in the NAcc core. (* indicates statistical significance ($p < .05$) relative to control group).

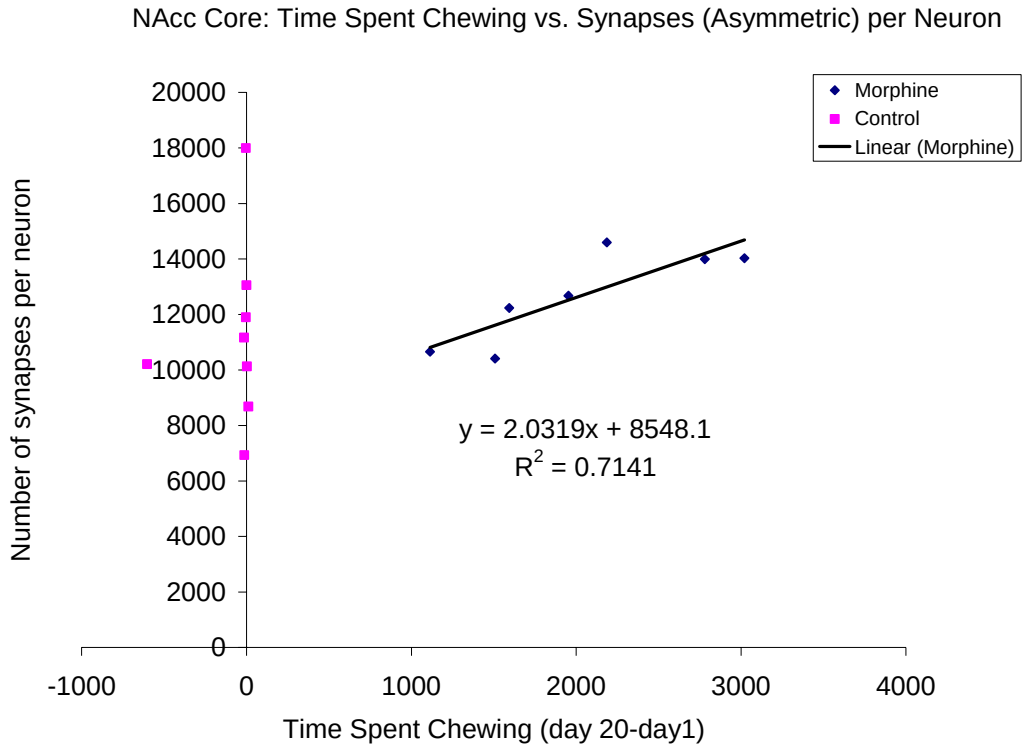

Figure 5.8. Time spent chewing versus the number of asymmetric synapses per neuron in the NAcc core of morphine-sensitized animals. A correlation analysis suggests a direct positive correlation ($R^2 = 0.7141$) between the number of asymmetric synapses per neuron in the NAcc core and the degree of sensitization (the difference in the time spent chewing between the last and first day of drug administration).

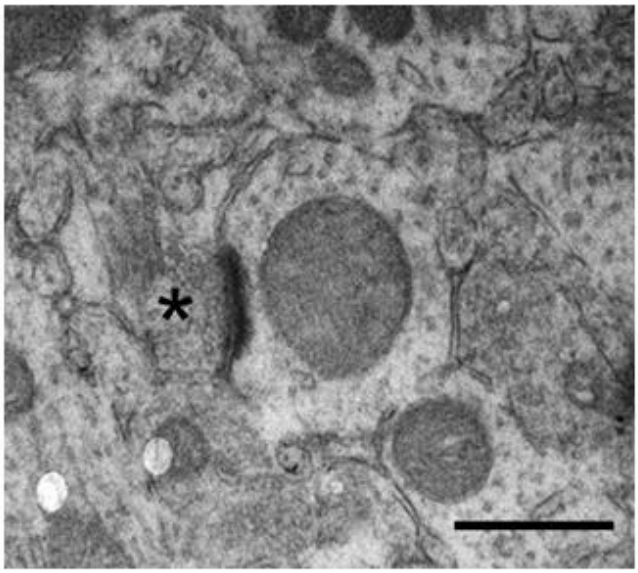

Figure 5.9. Asymmetric synapse onto a dendritic shaft in the NAcc shell. Electron micrograph (18,000X) taken of an asymmetric synapse onto a dendritic shaft within the NAcc shell of a morphine-treated rat (Scale bar = 500 nm). (* indicates asymmetric axon terminal contacting dendrite).

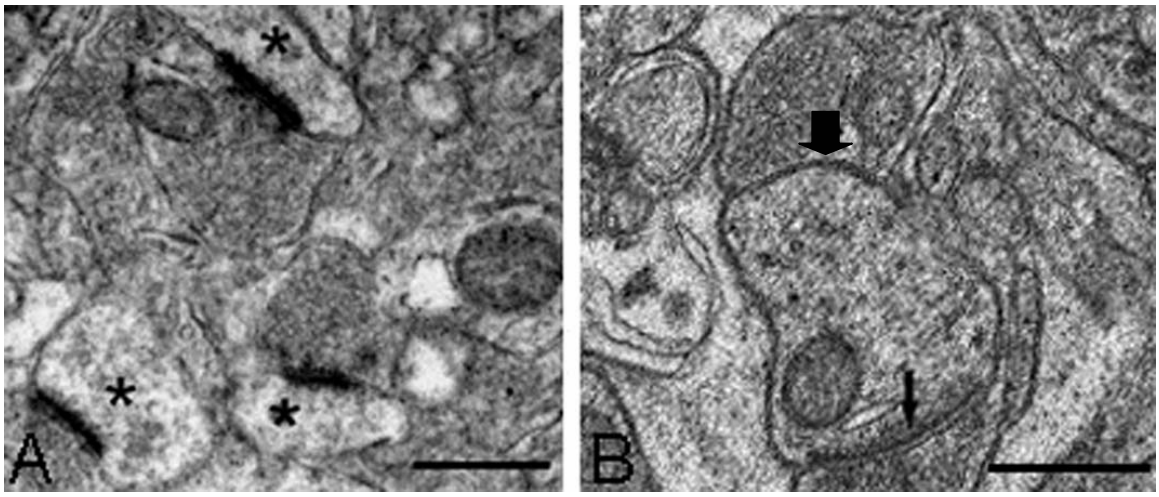

Figure 5.10. Asymmetric and symmetric synapse onto a dendrite in the NAcc core. Electron micrographs (18,000X) taken of an asymmetric synapse onto a dendritic spine (asterisk) of a cocaine-treated rat (A) and dendritic shaft (thin arrow) of a control rat (B) within the NAcc core. A symmetric synapse (wide arrow) also appears in B making contact with the dendritic shaft (Scale bars = 500 nm).

Table 5.1 Percentage of Asymmetric and Symmetric Synapses onto Dendritic Spines and Dendritic Shafts in the Nucleus Accumbens Shell and Core

	Control	Cocaine	Morphine
Shell			
Total Number of Synapses per Neuron	7759 ± 1286	11567 ± 2862*	12036 ±3363*
Dendritic Spine	42.1% ± 4.4%	34.0% ± 4.4%	41.7 % ± 4.2%
Dendritic Shaft	44.3% ± 4.5%	53.5% ± 4.5%	42.8% ± 4.2%
Asymmetric	71.1% ± 2.9%	77.5% ± 3.1%	75.9% ± 3.1%
Dendritic Spine	54.1% ± 5.3%	49.5 % ± 5.7%	43.3% ± 5.7%
Dendritic Shaft	33.7% ± 4.8%	39.0% ± 5.2%	45.9% ± 5.2%
Symmetric	23.5% ± 2.5%	18.5% ± 2.6%	20.8% ± 2.6%
Dendritic Spine	9.9% ± 5.1 %	18.4% ± 5.5%	9.8% ± 5.5%
Dendritic Shaft	80.6% ± 5.2%	74.7% ± 5.5%	86.8% ± 5.5%
Core			
Total Number of Synapses per Neuron	15781 ± 3982	22477 ± 5877*	15645 ± 2083
Dendritic Spine	43.2% ± 3.1%	39.3% ± 3.1%	42.8% ± 2.9%
Dendritic Shaft	26.8% ± 2.9%	32.4% ± 2.9%	29.1% ± 2.7%
Asymmetric	71.2% ± 3.5%	72.1% ± 3.7%	73.9% ± 3.7%
Dendritic Spine	53.8% ± 3.2%	55.3% ± 3.4%	48.7% ± 3.4%
Dendritic Shaft	22.8% ± 1.9%	20.2% ± 2.0%	25.8% ± 2.0%
Symmetric	24.2% ± 3.5%	21.8% ± 3.7%	20.1% ± 3.7%
Dendritic Spine	11.3% ± 3.2%	11.5% ± 3.2%	18.1% ± 3.0%
Dendritic Shaft	56.4% ± 6.8%	67.7% ± 6.8%	50.5% ± 6.4%

Table 5.1. Percentage of Asymmetric and Symmetric Synapses onto Dendritic Spines and Dendritic Shafts in the Nucleus Accumbens Shell and Core. (The values listed reflect the total group means and standard errors for each condition (Control, Cocaine, Morphine), the percent of total synapses categorized as asymmetric and symmetric that made contact with either dendritic spines or dendritic shafts). * indicates significance at $p < .05$.

5.5 Discussion

The present study showed that 1) rats chronically administered cocaine or morphine (4 weeks) exhibit behavioral sensitization, and that following 3 weeks of drug abstinence, synaptic reorganization is observed in the NAcc shell, evident by an increase in the number of synapses per neuron, 2) these synaptic changes in the NAcc shell of drug-treated animals are driven by alterations in the number of asymmetric, presumably glutamatergic excitatory, synapses and 3) only cocaine-sensitized animals showed synaptic rewiring of asymmetric synapses in the NAcc core.

In the present study, a categorical shift (i.e. locomotor to stereotyped) in the expression of cocaine-induced behaviors was observed between the first and last days of drug administration. Behavioral sensitization to cocaine was observed through the analysis of drug-induced stereotyped behaviors, including headbobbing and sniffing, but not the locomotor activating effects of cocaine, such as line crossing and rearing. Such changes in drug-induced behaviors over time have been previously described as the U-shaped function (Fig. 5.11) relating psychostimulant dose to performance (Lyon and Robbins 1975; Dews and Wenger 1977). This theory describes a shift in the expression of drug-induced motor behaviors as drug dose varies. For example, both low and high doses of cocaine increase the behavioral motor response in a rat, but the low dose induces more line crosses while the high dose induces more stereotypic headbobbing. Thus, this shift is characterized by the disappearance of exploratory behaviors and replaced by the emergence of repetitive, purposeless stereotypic behaviors. Additionally, the design of the testing apparatus can influence the behaviors expressed by animals. For instance, Robinson and Kolb (1999b) placed a box insert in the middle of the test cage to create a walkway along the perimeter of the testing apparatus for the rats to move around following drug treatment. As such, sensitization was found through analysis of locomotor

activity, comparing the first and last day of drug treatment. The expression of sensitization in the present study found through analysis of stereotypic behaviors may have been influenced by the design of the testing apparatus which allowed for the expression of both locomotor and stereotypic behaviors. Previous studies have implicated NMDA receptors in the induction of cocaine sensitization because blockade of this receptor, using MK-801, during pretreatment with cocaine prevents the induction of behavioral sensitization (Wolf and Jeziorski, 1993). Moreover, application of this NMDA receptor antagonist also prevents certain molecular neuroadaptations associated with cocaine sensitization, such as DA autoreceptor subsensitivity and DA D1 receptor supersensitivity (Li et al., 1999b).

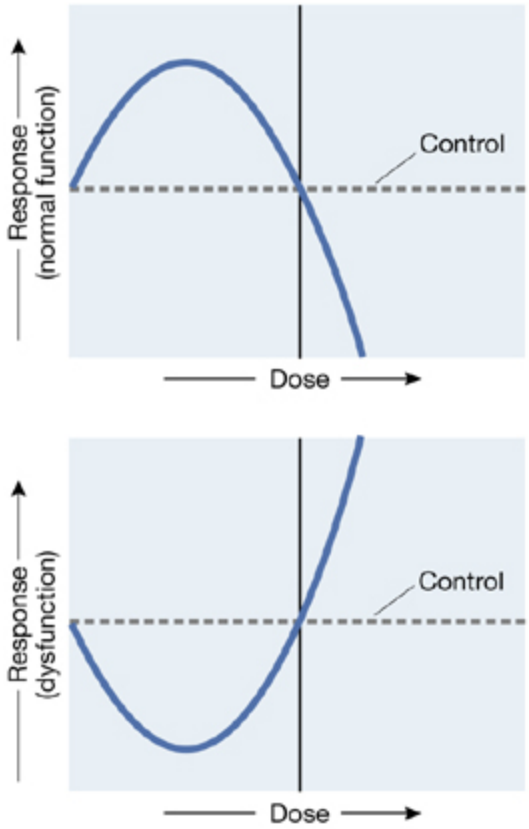

Figure 5.11 The inverted U-shaped model. These graphs depict the shift in behavioral response as dose varies. (A) Normal function applies to the increase in line crosses and exploratory behaviors observed with low doses of cocaine and the disappearance of these behaviors at higher cocaine doses. (B) Dysfunction applies to the emergence of stereotypic behaviors (repetitive, purposeless behaviors) that increase as drug dose increases. This figure was adapted from (Davis and Svendsgaard, 1990).

Morphine administration, under this particular drug regimen, produced both motor sensitization (oral stereotypy) and motor tolerance (opiate catalepsy). Morphine-induced stereotyped chewing/gnawing behavior has received little attention (Pollock and Kornetsky, 1989; Di Chiara, 1999), thus the neurochemical mechanisms underlying morphine-induced oral stereotypy are not yet clear. Fluoxetine, MK-801 and SCH23390 all block the expression of this behavior, and only pretreatment with MK-801, an NMDA receptor antagonist, blocked the development of sensitization to this oral stereotypy

(Livezey et al., 1995; Wennemer and Kornetsky, 1999), thus, indicating an important role for glutamate in the development of this particular type of morphine-induced sensitization.

Morphine-induced motor tolerance, as measured by morphine-induced catalepsy, has been reported in only a few studies to date (Hand and Franklin, 1985). The exact mechanism underlying opiate-induced catalepsy is not known, although it can be significantly prevented or reversed by phenytoin and naloxone (Cookson and Mann, 1980). Morphine-induced immobility proved to be a reliable measure of motor tolerance in this study. This finding may be useful for future opiate tolerance studies, not necessarily interested in the analgesic effects of morphine, because it does not require a separate testing apparatus or added stressors (i.e. submersion of the tail in hot water) as is the case for the more commonly used tail-flick test.

Both cocaine and morphine administration produced increases in the number of asymmetric synapses in the NAcc shell. First, this neuroadaptive effect shared by both drugs is in accordance with evidence surrounding the function of the NAcc shell, which is believed to be more responsive to reward signals because of its dense innervation by the limbic system. This hypothesis is further supported by the observation that DA release in the NAcc shell does not undergo habituation following repeated administration of drug-rewards, which is the case with non-drug rewards, such as caffeine (Di Chiara, 1999). Thus, the synaptic changes observed in the NAcc shell reported in this study may be a result of a common reward-mechanism utilized by both drugs. Secondly, at the microcircuit level, an increase in the number of asymmetric synapses (presumably glutamatergic) in the NAcc shell suggests that projections from the PFC, amygdala, hippocampus, or thalamus may be occurring. The increase in the number of synapses per neuron in the NAcc shell is consistent with the increase in spine density reported by

Robinson and Kolb (1999b) in a cocaine study using a similar drug regimen. In the present study, analysis of the percentage of asymmetric synapses onto a specific postsynaptic target in the shell of cocaine-treated animals did not reveal any specific trends (i.e. synapse onto dendritic spine vs. synapse onto dendritic shaft) relative to controls. Thus, a more specific analysis is needed to determine the source and target of the synaptic changes associated with chronic cocaine treatment.

In morphine-treated animals, an overall increase in the number of asymmetric synapses per neuron in the NAcc shell occurs despite the reduction in spine density as noted by Robinson and Kolb (1999a). Dendritic spine density is unaltered in the NAcc core following repeated morphine administration (Spiga et al., 2005), which is consistent with the findings of the present study. Interestingly, a trend towards an increase in the number of asymmetric synapses onto the dendritic shaft (versus a dendritic spine) suggested that this category of synapse may be more prevalent in this brain region. This trend is worth noting because this evidence suggests that 1) the decrease in spine density may not result in the loss of synaptic connections, rather it could be that the synapses retain contact with the target cell and the functional differences between synapses onto the dendritic shaft versus spine head and the differences in their effects on membrane excitability become important for this structural change and drug-induced behaviors. However, there is not enough information in this study to support this theory, and would have to be directly examined in a future study.

Cocaine-sensitized rats revealed increases in the number of asymmetric synapses in the NAcc core. This finding agrees with previous studies depicting the NAcc core as a structure integrating motor with behavior. For example, cocaine-induced structural changes have been shown to occur in the NAcc core in animals expressing behavioral sensitization (Li et al., 2004), and lesions of the NAcc core severely impair cocaine-

seeking behavior (Ito et al., 2004). In addition, the increase in the number of excitatory synapses in this brain region supports the idea of strong glutamatergic involvement in the mechanisms underlying behavioral sensitization to cocaine. Finally, the synaptic changes in the NAcc core were specific to the cocaine-sensitized, but not morphine-sensitized animals. Pharmacological and electrophysiological studies examining the effects of cocaine on behavioral sensitization consistently show involvement of the PFC. For instance, cocaine increases extracellular glutamate levels in the NAcc (Smith et al., 1995), but morphine inhibits its release (Sepulveda et al., 1998). Similarly, repeated electrical stimulation of the PFC produces behavioral sensitization (Schenk and Snow, 1994), whereas lesions of the PFC block the development of this behavior (Tzschentke and Schmidt, 1998). A separate analysis is necessary to determine whether the changes in the number of asymmetric synapses per neuron are in fact occurring within glutamatergic microcircuits associated with the PFC, because other glutamatergic projections to the NAcc arise from the amygdala, hippocampus and thalamus. Such changes in synaptic connectivity presumably occur as a consequence of repeated drug treatment and persist even after drug administration ceases; however such a conclusion requires a subsequent time course study in which synaptic changes are measured immediately following drug cessation and after an extended period of abstinence.

A correlation analysis comparing the degree of sensitization (comparing first and last day of drug treatment) of morphine-treated animals and the number of asymmetric synapses per neuron in both the shell and core compartments of the NAcc revealed a marginally strong negative correlation ($R^2 = 0.7332$) in the NAcc shell, and interestingly, a positive correlation ($R^2 = 0.7141$) in the NAcc core. Reciprocal changes for the NAcc shell and core have been previously reported in morphine-sensitized animals (Cadoni and Di Chiara, 1999). Although Cadoni and Di Chiara (1999) report reciprocal changes in

DA responsivity of the NAcc shell and core, because of the well-established convergence of DA and glutamate onto MSNs (Sesack and Pickel, 1992; Kotter, 1994), the present findings may be a neuroadaptive change in response to the change in DA as a result of repeated morphine administration. This would have to be examined directly in a future study, likely with a higher number of animals in each group to detect a stronger correlation if one exists.

The behavioral effects of repeated cocaine and morphine treatment in the present study produced sensitization in markedly different stereotypic behaviors, for example headbobbing and stereotypic sniffing (cocaine) versus chewing gnawing (morphine). These findings suggest that psychostimulants and opiates engage different neuronal mechanisms in behavioral sensitization, and such findings have been reported in a previous cross-sensitization study examining stereotypic behaviors exhibited by morphine-sensitized animals that receive a challenge injection of cocaine (Cadoni and Di Chiara, 1999). Moreover, results from the correlation analysis in the present study revealed two marginal correlations in morphine-sensitized animals. These correlations were opposite in direction (negative correlation in the shell and positive correlation in the core) between the number of asymmetric synapses quantified in the NAcc shell and core and the degree of sensitization. Evidence from the correlation analysis, although only marginal, suggests reciprocal functions of the shell and core compartments of the NAcc in morphine sensitization, which has been previously suggested. Reciprocal changes in DA neurotransmission in the shell and core compartments of the NAcc has been previously reported, where DA levels were shown to decrease in the shell and increase in the core following repeated morphine treatment (Cadoni and Di Chiara, 1999). Thus, the decrease in the number of asymmetric, possibly glutamatergic, synapses in the shell and increase in the core reported in the present study may be a consequence of changes in DA

release, given the convergence of dopaminergic and glutamatergic terminals on MSNs of the NAcc (Sesack and Pickel, 1992). Furthermore, no correlation was found in either brain region with cocaine-sensitized animals, further supporting the notion that different mechanisms underlie behavioral sensitization to cocaine and morphine. Additional cross-sensitization studies are needed in order to further differentiate the long-term behavioral sensitizing effects, and the synaptic neuroadaptations accompanying these persistent behavioral changes, of different drug classes, such as psychostimulants and opiates.

In conclusion, the present study reveals that long-term treatment with cocaine or morphine induces synaptic reorganization by increasing the number of excitatory synapses in the NAcc shell, and that similar changes in connectivity are observed in the NAcc core in cocaine-sensitized animals. To determine whether the neuroadaptive synaptic changes in the NAcc, reported in the present study, are causally-related to the observed behavioral changes, subsequent studies are needed. For example, additional studies aimed at abolishing behavioral sensitization through the blockade of drug-induced spine density changes, for example with the use of roscovitine, which has been shown to prevent cocaine-induced spine proliferation, could be conducted. Moreover, an examination of the contribution of local circuit neurons and specific brain regions projecting the NAcc that may be contributing to the increase in the synapse per neuron ratio also requires future analysis. The present study identifies a specific neuroadaptive change, synaptic plasticity, associated with long-term drug use. Such changes in synaptic connectivity found within the NAcc, a primary target of the mesolimbic DA pathway and drugs of abuse, are the first evidence of persistent neuroadaptive changes that could potentially underlie behavioral sensitization and other drug-related behaviors. Together these data suggest an important role for glutamate neurotransmission in the maintenance of long-term neuroadaptive changes and persistent behavioral changes observed during

drug abstinence. Furthermore, these findings offer new targets, such as specific cell types or synaptic circuits for future pharmacological and behavioral therapies for the prevention and treatment of drug abuse and dependency.

Chapter 6: General Discussion and Future Directions

Taken together, the four studies comprising this dissertation examined the effects of acute and chronic drug administration on the NAcc shell and core, with particular emphasis on specific cellular circuits and synaptic plasticity potentially involved in drug abuse and dependence. Specifically, these studies focused first on the cholinergic interneurons of the NAcc shell, a region known to respond, metabolically, neurochemically and molecularly, during the initial stages of drug use. Despite the major role of the NAcc in drug administration studies, the specific contribution of the cholinergic interneurons of the NAcc has received little attention in the field of drug abuse and dependency. Therefore, the question addressed in the first study (chapter 2) was whether these neurons were involved following acute (i.e. first-time) drug administration. This study showed that these cholinergic interneurons were dose-dependently activated following the acute self-administration of cocaine, specifically in the shell compartment of the NAcc, revealing a potential role for this particular cell population in drug abuse.

The second and third studies (chapters 3 and 4) tested whether these cholinergic neurons expressed key excitatory (DA D5) and inhibitory (DA D2) receptor subtypes. Administration of drugs of abuse leads to changes in DA transmission within the NAcc following short and long-term drug treatment. Dopamine receptors are likely activated by drug-induced changes in extracellular DA levels, as well as drug-induced behavioral changes, such as cocaine-induced conditioned place preference (Graham et al., 2006). Although previous studies have shown that cholinergic cells in the NAcc express high levels of both DA D5 and DA D2 receptor mRNA, the localization of the receptor protein within this cell population was unknown. These studies localized both DA receptor

subtypes at specific locations along the cell bodies, dendrites and axons of cholinergic interneurons in the NAcc. The specific neuroanatomical localization of these receptors provided insight into two possible molecular substrates (i.e. DA D5 and DA D2 receptors) mediating dopaminergic influences of ACh neurotransmission in the NAcc. Dopamine receptor neuroadaptations in the NAcc were also investigated, using a repeated 2-week drug regimen, in rats trained to lever press for intravenous cocaine infusions (i.e. cocaine self-administration paradigm), but due to some limitations in the study (for example, animals did not self-administer enough cocaine), the findings proved inconclusive. Thus, the potential effects of repeated drug-treatment on DA D5 and DA D2 receptor neuroadaptations remain to be examined in a future study.

Dopamine activation of cell surface receptors, following drug administration, leads to the induction of intracellular cascades, involving mechanisms (e.g. CREB, delta FosB, CDK5, etc.) which have been linked to persistent cellular and morphological changes in the brain, presumably underlying long-lasting drug-induced behavioral changes; however, changes in synapse number, as a result of repeated drug treatment, had not been directly examined. Therefore, the fourth study (chapter 5) investigated whether synaptic alterations occur in the NAcc following long-term drug treatment. This final study demonstrated, for the first time at the electron microscopic level, that chronic (4-week) treatment with cocaine or morphine leads to synaptic rewiring in the NAcc, and is persistent following 3 weeks of drug-abstinence. More specifically, an increase in the number of excitatory synapses in the NAcc shell occurs with both psychostimulants and opiates, and similar synaptic reorganization occurs in the NAcc core specifically in cocaine-sensitized animals. Future work remains to determine more specifically whether the synaptic changes are a direct result of the drug administration itself or the combined effects of repeated drug treatment and drug abstinence.

In all, findings from these studies are in agreement with previous reports supporting a significant role for the NAcc as a key neuroanatomical substrate involved in acute and chronic drug administration, and further identify specific potential neural substrates and sites of synaptic plasticity. Additional studies are needed to examine the neuroadaptive effects of drug administration on DA receptor expression and long-lasting synaptic changes within cholinergic interneurons of the NAcc shell and core. Such studies, which elucidate the specific contribution of discrete cell populations in critical brain areas targeted by drugs of abuse, are rare, but are necessary for the development of more specific drug treatments aimed at combating drug abuse and dependence. Furthermore, evidence of drug-induced increases in the number of excitatory (presumably glutamatergic) synapses within the NAcc requires further investigation of possible sources of excitatory inputs, such as the PFC, hippocampus, amygdala, or thalamus. Similarly, the convergence of glutamatergic and dopaminergic inputs onto MSNs would more clearly address the question of the increasingly ambiguous role of DA in reward and reinforcement while shedding light on the combined effects of DA and glutamate in associative-learning mechanisms posited to underlie cellular processes in addiction. Such neuroadaptations, if examined in future studies, will aid in the development of new therapies and pharmaceutical treatments designed to combat persistent drug-induced behaviors, such as compulsive drug intake, tolerance, withdrawal, drug craving and relapse.

6.1 Conclusion

These studies show that cholinergic cells in the NAcc shell are involved in the initial stages of cocaine intake, and further suggest that DA D5 and D2 receptors expressed on these cells and their potential neuroadaptation can potentially mediate the response of these cells to drug-induced increases in DA neurotransmission in the NAcc;

however, direct evidence of a role for DA receptor neuroadaptation on cholinergic neurons in addiction remains to be examined. Furthermore, the final study provides the first ultrastructural evidence that 4 weeks of repeated cocaine (psychostimulant) and morphine (opiate) treatment followed by 3 weeks of abstinence leads to 1) an increase in the number of excitatory synapses in the NAcc shell and 2) cocaine sensitization, as measured by drug-induced stereotypic behaviors, is associated with an increase in the number of excitatory synapses in the NAcc core; however future work will determine whether the synaptic rewiring is due to the drug treatment itself or the combination of repeated drug administration followed by drug cessation. The specific mechanisms through which these drug-induced synaptic changes occur is not known; however, continued work on the molecular and cellular forms of neuroplasticity in conjunction with future synaptic and behavioral analyses will reveal additional information as to the key neuronal substrates underlying drug-induced changes in the brain and behaviors. Such studies are critical to the understanding, treatment and ultimate prevention of drug abuse and dependency.

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